Diversity of the Genus *Bacillus* and the Occurrence of Bacterial Endospores in Soil, with Special Reference to *Bacillus anthracis*

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Summary

This work originated from the anthrax contaminated mail in the USA in 2001 and the subsequent hoax and threatening letters of copycats all over the world. Anthrax, a long-forgotten infectious animal disease, caused by the endospore forming bacterium *Bacillus anthracis*, once was relatively widespread. It is zoonotic, that means, that it can be transmitted to people. Specific sites could still be contaminated with viable spores and serve as natural reservoirs. Therefore the responsible authorities of the canton of Zurich wanted to have an overview of these “natural” anthrax contaminations. This led to **Chapter I** of this thesis: The historical investigations about anthrax in the canton of Zurich and the land register of possibly contaminated locations. Historical records reporting cases of animal anthrax in the canton of Zurich between 1878 and 2005 were analyzed on the level of political communities regarding occurrence and number of cases, animals affected, and number of communities affected. Unfortunately information on human cases was not available anymore. Data were correlated with industrial activities (tanning, wool and horse hair processing) in a community and to the prevailing meteorological conditions. A total of 830 cases of animal anthrax has been recorded in 140 of 171 communities. Occurrence correlated with industrial activities in a community such as companies handling potentially contaminated materials (hides, fur, wool, hair, meat, or bone meal). The influence of wool processing companies ($P = 0.004$) and tanneries ($P = 0.032$) was significant whereas horse hair processing had no effect. However, a statistical relationship between the number of cases reported and meteorological data (rainfall, mean temperature) was not found. Possibly contaminated locations are (former) industrial sites of companies with above mentioned anthrax-relevant activities, carcass disposal sites as well as farms and pastures where anthrax infections occurred. For the canton of Zurich a considerable number of such sites was identified. All sites were assessed regarding the probability of contamination and the actual infection risk. Most sites were determined to present no actual risk, some may be critical and a few are critical.

The aim of the **Chapter II** of this work was to evaluate and (further) develop fast, easy and inexpensive methods to detect bacterial spores, in particular *B. anthracis*. Non-invasive physico-chemical methods were determined to be the most adequate approach. First, a fluorescence method was developed. Dipicolinic acid (DPA) is a chemical component exclusively occurring in bacterial endospores. In solution DPA forms a fluorescent chelate complex with terbium ions. This fact is the basis of the method. DPA was released from spores by microwaving or autoclaving. The strength of the fluorescence signal corresponds to the amount of DPA available in a sample and therefore indicates the amount of spores. Soil from different sites (grassland, garden, forest and aquatic sediments) was analyzed. The highest spore content (up to 10^{10} spores per gram of dry soil) was found in soil from grasslands and in soil depths between 5 and 10 cm. As second Fourier transform infrared (FTIR) spectroscopy was tested and an applicable protocol designed. An IR

spectrum reflects the sum of all movements of the molecules and atoms in a sample excited by an infrared beam. Spectra are dependent on the chemical components and for this reason specific for a sample. Hence FTIR can be applied to detect and identify microorganisms. Endospores from pure cultures of seven different *Bacillus* species (*B. atrophaeus, B. brevis, B. circulans, B. lentus, B. megaterium, B. subtilis, B. thuringiensis*) in the presence of different solid matrix substances such as clay minerals (bentonite), milk powder, powdered mushrooms or dry herbs as well as mixtures of matrix compounds and bacterial strains at various wt/wt ratios have been investigated. It was possible to discriminate different *Bacillus* species in pure cultures and spores-matrix-mixtures with simple matrices. Soil samples could not be discriminated.

In **Chapter III** selective media were tested for direct use with soil suspensions. These were the well known selective PLET agar, recommended by WHO, and the recently developed anthracis chromogenic agar. Samples were taken from a former carcass disposal site, where animals died from anthrax have been buried. However, no viable *B. anthracis* spores were found.

Chapter IV, the last part of this thesis was the assessment of bacillus diversity in soil by molecular methods. A protocol was developed using polymerase chain reaction and temporal temperature gradient electrophoresis, PCR-TTGE fingerprinting technique and a *Bacillus*-specific primer pair for 16S rDNA. Samples were collected from a grassland site along a transect and at different depths. A total of 18 distinguishable bands was found. As a general rule one band represents one species. The community was found to be significantly influenced by the sampling point ($P<0.001$) and to a minor extent by the content of organic carbon ($P<0.01$). Sampling depth showed no significance.

Zusammenfassung

Diese Arbeit entstand aufgrund der Anthrax-Briefe in den USA im Jahre 2001 und der Scherz- und Drohbriefe von Trittbrettfahrern, die darauf in der ganzen Welt auftauchten. Anthrax (Milzbrand), eine vergessene Tierkrankheit, verursacht durch das Endosporen-bildende Bakterium *Bacillus anthracis*, war einst relativ weit verbreitet. Es handelt sich um eine Zoonose, d.h. auch Menschen können sich anstecken. Verschiedene Standorte könnten noch immer mit keimfähigen Sporen kontaminiert sein und so als natürliche Reservoire dienen. Aus diesem Grund wollten sich die zuständigen Behörden des Kantons Zürich eine Übersicht verschaffen über solche „natürlichen“ Anthrax-Kontaminationen, die ein gewisses „Anthrax-Grundrauschen“ darstellen. Dies führte zum **Kapitel I** dieser Doktorarbeit: Historische Recherchen über Anthrax im Kanton Zürich und der Kataster von möglicherweise kontaminierten Standorten. Historische Aufzeichnungen über Milzbrandfälle bei Tieren im Kanton Zürich zwischen 1878 und 2005 (Tierseuchenregister) wurden analysiert, wobei das Auftreten und die Anzahl von Fällen, die erkrankten Tierarten und die Anzahl betroffener politischer Gemeinden untersucht wurden. Informationen über humane Fälle waren leider nicht mehr verfügbar. Die Daten wurden sowohl mit den industriellen Aktivitäten (Gerben, Woll- und Rosshaarverarbeitung) in den Gemeinden als auch mit den damals vorherrschenden meteorologischen Bedingungen korreliert. In der Untersuchungsperiode wurden insgesamt 830 Milzbrandfälle bei Tieren in 140 von 171 Zürcher Gemeinden verzeichnet, wobei mehrheitlich Rinder betroffen waren. Das Auftreten der Fälle korrelierte mit industriellen Aktivitäten in der jeweiligen Gemeinde. Ein positiver Zusammenhang zwischen dem Auftreten von Milzbrandfällen in einer Gemeinde und dem Vorhandensein lokaler Unternehmen, die potenziell kontaminiertes Material wie Häute, Felle, Wolle, Haar, Fleisch oder Knochenmehl verarbeiteten, konnte gezeigt werden. Der Einfluss von wollverarbeitenden Betrieben ($P = 0.004$) und Gerbereien ($P = 0.032$) erwies sich als erheblich, während rosshaarverarbeitende Betriebe keinen Einfluss hatten. Zwischen dem Auftreten von Milzbrandfällen und meteorologischen Bedingungen (Niederschlag, Temperatur) gab es keinen statistisch signifikanten Zusammenhang. Möglicherweise kontaminiert sind (frühere) Standorte von oben erwähnten Anthrax-relevanten Betriebszweigen, Wasenplätze (Plätze, wo Kadaver vergraben wurden) sowie Höfe und Weiden, wo Anthraxinfektionen aufgetreten sind. Im Kanton Zürich konnte eine beträchtliche Anzahl solcher Orte eruiert werden. Für alle Standorte wurde die Wahrscheinlichkeit einer tatsächlichen Kontamination und das aktuelle Infektionsrisiko abgeschätzt. Die meisten wurden als ungefährlich eingeschätzt, einige als möglicherweise kritisch und wenige als kritisch.

Ziel des **Kapitel II** dieser Arbeit war es, schnelle, einfache und kostengünstige Methoden zur Detektion und Identifizierung von bakteriellen Sporen, insbesondere *B. anthracis*, zu evaluieren und ggf. (weiter) zu entwickeln. Nicht-invasive physikalisch-chemische Methoden schienen dazu der geeignetste Ansatz zu sein. Als Erstes wurde eine Fluoreszenz-Methode entwickelt.

Dipicolinsäure (dipicolinic acid, DPA) kommt ausschliesslich in Bakteriensporen vor. In Lösung bildet es mit Terbiumionen einen fluoreszierenden Chelat-Komplex. Diese Gegebenheit bildet den Kern der Methode. DPA wurde mittels Mikrowellenbehandlung oder Autoklavieren aus den Sporen freigesetzt. Die Fluoreszenzstärke entspricht der Menge an DPA in der Probe und somit gibt sie Aufschluss über die Sporenmenge. Boden von verschiedenen Standorten (Wiesland, Garten, Wald und aquatisches Sediment) wurde untersucht. Den höchsten Sporengehalt (bis 10^{10} Sporen pro Gramm Boden, Trockengewicht) wiesen Bodenproben aus einer Tiefe von 5 bis 10 cm von Wiesland auf. Als zweite Methode wurde Fourier Transformation Infrarot (FTIR) Spektroskopie getestet und ein entsprechendes Protokoll erstellt. Ein IR Spektrum gibt die Summe aller Bewegungen der Moleküle und Atome einer Probe wieder, die durch einen Infrarotstrahl angeregt werden. Die Spektren hängen von der chemischen Zusammensetzung der Probe ab und sind daher sehr spezifisch. Darum kann FTIR zur Detektion und Identifizierung von Mikroorganismen eingesetzt werden. Analysiert wurden Sporenpulver aus Reinkulturen von sieben verschiedenen *Bacillus*arten (*B. atrophaeus, B. brevis, B. circulans, B. lentus, B. megaterium, B. subtilis, B. thuringiensis*), verschiedene feste Matrixsubstanzen, wie Tonmineralien (Bentonit), Milchpulver, Champignonpulver oder getrocknete Kräuter, sowie Mischungen aus Matrix und Bakterienstämmen in unterschiedlichen Gewichtsverhältnissen. Verschiedene *Bacillus* Arten als Reinsubstanz und Sporen-Matrix-Mischungen mit einfacher Matrix konnten unterschieden werden. Bei Bodenproben war keine Unterscheidung möglich.

In **Kapitel III** wurden selektive Nährmedien für den direkten Gebrauch mit Bodensuspension getestet. Es waren dies der bekannte selektive „PLET Agar“, der auch von der WHO empfohlen wird, und der erst vor einigen Jahren entwickelte „Anthracis chromogene Agar“. Als Proben diente Boden von einem ehemaligen Wasenplatz, wo an Anthrax verendete Tiere vergraben worden sind. Es wurden keine keimfähigen Sporen gefunden.

Kapitel IV, der letzte Teil dieser Doktorarbeit, ist der Diversität von *Bacillus* im Boden gewidmet, erfasst mittels molekularbiologischer Methoden. Es wurde ein Methoden-Protokoll entwickelt unter Anwendung der Polymerase-Kettenreaktion und Temperaturgradienten-Elektrophorese, PCR-TTGE-Fingerprint-Technik und einem *Bacillus*-spezifischen Primerpaar für die 16S rDNA. Die verwendeten Bodenproben einer Wiese entnommen, entlang eines Transsekts und aus verschiedenen Tiefen. Gesamthaft wurden 18 unterscheidbare Banden gefunden. In der Regel entspricht eine Bande einer Art. Die Analyse zeigte einen signifikanten Einfluss des Probenahmepunktes auf die *Bacillus*-Gesellschaft ($P<0.001$). Die Beeinflussung durch den Gehalt an organischem Kohlenstoff war etwas weniger ausgeprägt ($P<0.01$). Die Tiefe hatte keinen Einfluss.

Introduction

Introduction

General Introduction

Bacteria are ubiquitous and found nearly everywhere in the world even under most extreme or worst conditions. They developed different strategies and mechanisms to live and survive in habitats such as hot deep-sea wells, glaciers, acidic lakes, or the mammalian guts for example. One survival mechanism of certain microorganisms is the formation of spores when exposed to unfavorable environmental conditions (e.g. nutrient limitation). This dormant state allows a temporal and/or spatial escape from the actual local conditions (Nicholson *et al.* 2000). In 1676 Antoni van Leeuwenhoek observed and described bacteria for the very first time (van Leeuwenhoek 1932). Although bacterial endospores have only been discovered 200 years later in the 1870ies independently by Cohn, Koch, and Tyndall (Cohn 1876; Koch 1876; Tyndall 1877). It is assumed that only about one to five percent of the existing bacteria have been discovered so far. Most of the known bacteria are harmless, some are beneficial (e.g. intestinal flora,) and some are responsible for more or less severe diseases (e.g. anthrax, cholera, plague, salmonellosis, tuberculosis, respiratory infections).
The best investigated bacteria belong to the endospore-forming genus *Bacilus* with the famous model organism *B. subtilis*. But most research, especially concerning endospores, is still done in the laboratory (Nicholson *et al.* 2000; Nicholson 2002). Therefore this study aimed to obtain some more information about *Bacillus* species and their endospores in a natural environment: Occurrence, frequency, and diversity in soil were investigated.

Bacillus anthracis

Organism

Bacillus anthracis is a Gram positive rod shaped, endospore forming soil bacterium, aerobic or facultatively anaerobic and approximately 4 µm by 1 µm (Turnbull *et al.* 1999). It is the causative agent of anthrax, an infectious disease with lethal potential for humans and animals. The spores are extremely resistant to chemical and/or physical environmental impact and are therefore able to survive in the environment for years or even decades and centuries to germinate and to form vegetative cells under appropriate, more favorable conditions (Nicholson *et al.* 2000). According to Titball *et al.* (1991), 200 year old anthrax spores have been found, which still were able to germinate. As beneficial for a long survival Dragon & Rennie (1995) mentioned soils rich in calcium and nitrate with neutral or slightly alkaline pH. Due to this extraordinary resistance endospores are the predominant form of this organism in the environment. Within the infected animal or human host, conditions favor the vegetative form, the bacterial spores germinate, the cells multiply and finally kill the host due to the release of toxins. While dying and after the host's death sporulation

starts because of less favorable conditions inside the host or because vegetative cells are shed into the environment. The lifecycle of *B. anthracis* (Fig. 1) is controversially disputed (Schmid & Kaufmann 2002). Van Ness (1971) hypothesized that under appropriate conditions the endospores germinate and replicate even in the environment. However, there are no scientific data available to support this hypothesis. Such conditions allowing germination might occur only very rarely in the environment. Furthermore, according to recent findings replication only occurs in the host. For this reasons multiplication requires an environment (soil) – host – cycle (Dragon & Rennie 1995) and, *B. anthracis* is an obligate pathogen (Turnbull *et al.* 1999).

Infection

The occurrence of anthrax is reported very early in history. The fifth plague mentioned in the Old Testament was probably anthrax (Missura 2001a). First and foremost, it is an animal disease, whereas primarily herbivores are affected. Carnivores and omnivores are more resistant (Watson & Keir 1994) and seem to be less at risk. Where the bacterium *B. anthracis* is located at the soil surface, animals can get infected with spores while grazing. Another source could be contaminated water or feedstuff for domestic animals. Humans are mostly infected directly or indirectly by animals or contaminated animal material (hides, fur, hair, bone meal, meat etc.). Therefore, it is mainly an occupational disease (agriculture, leather and skin production, hair and meat processing etc.) (Turnbull *et al.* 1999; Nicholson 2002; Pepper & Gentry 2002; Schmid & Kaufmann 2002; Anonym 2003). Person-to-person transmission is very uncommon (Watson & Keir 1994). There is

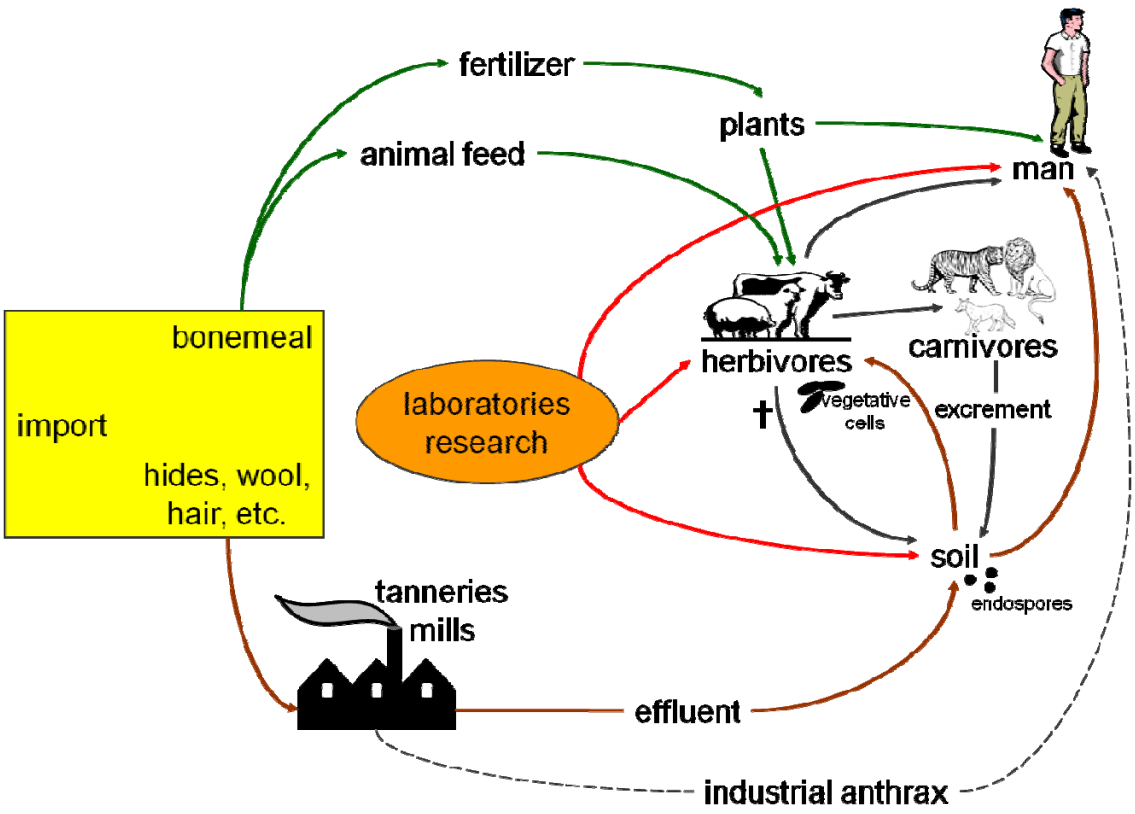

Fig. 1: Importation, spread, and cycles of *B. anthracis*

no risk of infection without direct contact to *B. anthracis* endospores. Besides the classification in **non-industrial** and **industrial** anthrax there are distinguished three types of the disease in humans: **Cutaneous** anthrax is the most innocuous and at the same time most common form (>95% of cases globally). Infection happens through mucous membranes and skin lesions, leads to itching inflammations and later to encrustation. For **gastrointestinal tract** anthrax lethality is higher. This form is acquired by ingestion of contaminated food or drink which causes inflammation of the throat and/or gastrointestinal tract. The most serious form is **pulmonary (inhalation)** anthrax, induced by inhaling respirable spores (as dust or aerosol). Symptoms are flu-like. Without treatment, or if it starts too late the infection is lethal. All three types are attended with high fever and cardiovascular problems. Development of meningitis is possible.

Occurrence

Until an effective veterinary vaccine was available and antibiotics were applied, animal anthrax was a worldwide problem (Turnbull *et al.* 1999). In several regions *B. anthracis* occurs naturally and according to World Health Organization WHO (Turnbull *et al.* 1999) and World Organization for Animal Health OIE (http://www.oie.int/hs2/report.asp) it is still reported periodically in:

- America: Central America, Mexico, Argentina, Guatemala, Peru, Bolivia, Venezuela, Haiti, single regions of Canada and USA.
- Africa: heavy incidence in equatorial African countries, Zimbabwe, Zambia, some cases in Botswana and South Africa.
- Europe: some Mediterranean countries (Spain, France, Italy, Greece, Turkey, former Yugoslavia), rarely Germany, single regions of Scandinavia, eastern Europe.
- Asia: middle Eastern and adjoining countries of former USSR, Pakistan, Nepal, southern India, Myanmar, Vietnam, Cambodia, western China, single regions of the Philippines and Indonesia.
- Australia: single regions.

For detailed information see appendix of this thesis.

In Switzerland bovine anthrax was relatively common until the sixties (Arbeitsgruppe Zoonosen 2002). Nowadays the disease occurs very rarely and as singular cases. The last major outbreak was in 1985 in the “Bündner Herrschaft”, where eleven cows and two goats died (Kuoni & Zindel 1986; Missura 2001b). The last animal case was reported in 1997 in the canton of Schwyz (BVET, http://www.infosm.bvet.admin.ch/public/?lang=de). The last human cases occurred between 1978 and 1985 in a textile factory in Flurlingen/Schaffhausen, where 25 workers have been affected (Pfisterer 1991). As in many other countries, the disease was introduced through import of contaminated animal raw materials. These are primarily hides and hair for the textile industry but also bone meal used as fertilizer and feedstuff (Fig. 1). Through strict surveillance, vaccination of animals, and disinfection of possibly contaminated raw material before import anthrax was nearly eradicated in Switzerland.

Historical cases of anthrax in Switzerland

It is not known to the public anymore, that anthrax is a natural occurring disease and even occurred regularly in Switzerland only 100 years ago. For this reason, the knowledge about ancient cases was lost, too. Even records from health and veterinary authorities are only rarely available because officially these reports have to be kept for maximally ten years only and they are often rudimental. Animal cases seem to be better documented than human cases. For human incidences we obtained some data from the Federal Office of Public Health and the Swiss Federal Statistical Office (statistic of causes of death), but they were not consistent. Data for animal cases were available from the national register of epizootics since 1900 and from some (handwritten) cantonal registers from the 19th century. In addition, there are only a few publications on animal anthrax in Switzerland (Kuoni & Zindel 1986; Sackmann 1994; Missura 2001b). Depending on the available data, only cases of animal anthrax in Switzerland and especially in the Canton of Zurich were further investigated. Results are given in Chapter I.

Detection methods

As mentioned before, bacteria are ubiquitous. Depending on their habitat it is more or less easy to collect and detect them. Bacterial spores are even more challenging than vegetative cells because of their dormancy, robustness and diminutiveness. Traditional detection methods are based on cultivation. Major drawbacks are the impossibility to detect non-culturable bacteria and these methods are often very time-consuming. More recent methods on the molecular level include the non-culturable but may still be time-consuming and usually are rather expensive. In most cases, preceding preparation steps include an extraction step which could pose problems depending on the matrix. Often there are some inhibitors in the sample which may interfere with one or more reactions of the method, for example humic acids in soil. Non-culturability, time consumption and costs may be overcome by non-invasive physico-chemical methods, such as fluorescence and infrared spectroscopy which are really fast, easy and inexpensive. But matrix and inhibitors may still be a problem. In liquid and air samples this could be solved by filtering. In solid samples normally the matrix or most part of it can not be removed and the more complex the matrix the more problematic the sample. Inhibitory compounds may be inactivated by addition of specific chemicals. The aim of our studies was to develop such non-invasive physico-chemical methods for the detection of bacterial spores in soil samples. Results are given in Chapters II and III.

Assessment of Bacillus diversity in soil

The genus *Bacillus* is one of the best investigated among bacteria. Many species belong to the soil bacterial flora and they form a large percentage of the culturable species in soil suspensions (Siala *et al.* 1974). In contrast to many laboratory studies, less is known on the occurrence, frequency,

and diversity of *Bacillus* species including their endospores in their natural soil environment. Most environmental investigations focus on specific species or on bacterial or even microbial diversity in general and in relation to vegetation and/or treatment of the sampling site (e.g. Kaiser & Heinemeyer 1993; Smalla *et al.* 2001; da Silva & Nahas 2002; Kuske *et al.* 2002; Nunan *et al.* 2002; Zhou *et al.* 2002; e.g. Agnelli *et al.* 2004; Horner-Devine *et al.* 2004). Only a few have a special look on *Bacillus* in general (e.g. Siala *et al.* 1974; Garbeva *et al.* 2003). As *Bacillus* was found to be an outstanding genus of the soil microbial community, at least regarding culturable species and biomass, it seems to be worth to investigate a) the diversity and distribution of *Bacillus* species in soil along a transect of a meadow formerly used as carcass disposal site and in different sampling depth and b) the relationship of soil parameters and the occurrence of different species. Results are given in Chapter IV.

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Chapter I

Bacillus anthracis in the canton of Zurich – a historical investigation

- Land register of possibly contaminated locations in the canton of Zurich
- 1st paper: Tier-Milzbrand in der Schweiz: Historische Fälle im Kanton Zürich zwischen 1878 und 1919; Vierteljahrsschrift der Naturforschenden Gesellschaft in Zürich (2006) 151 (4): 101–106
- 2nd paper: Milzbrand im Kanton Zürich zwischen 1878 und 2005; Schweizer Archiv für Tierheilkunde (2007) 149 (7): 295-300 (originally submitted in English)

Land register of possibly contaminated locations in the canton of Zurich

Abstract

As a result of the anthrax contaminated mail in the USA in 2001 and the following increased interest in anthrax, a land register of locations in the canton of Zurich possibly contaminated with *B. anthracis* was established. Sites were found through historical investigations, including interviews with communal offices and old people, looking for specific field and street names and consult old documents. An assessment of the probability of contamination and the actual infection risk was made for all locations. Finally, all data were processed by a geographical information system, GIS.

Introduction

Bacillus anthracis, the causative organism of anthrax, is a Gram-positive, endospore-forming soil bacterium. The spores are extremely resistant to environmental impact and are therefore able to survive in the environment for years or even decades and centuries (Titball *et al.* 1991; Nicholson *et al.* 2000). As anthrax once was a relatively widespread infectious animal disease, specific sites could still be contaminated with viable spores and serve as natural reservoirs (see also main introduction of this work). For example, in Germany *B. anthracis* was detected at some former tannery sites, associated wastewater treatment plants and along water drainage systems (Anonym 1999; Kautzky 2001). In Switzerland *B. anthracis* was found in soil from a former carcass burial site, six years after the last carcass has been buried (v. Gonzenbach 1915).

The anthrax contaminated mail in the USA in 2001 and the subsequent hoax and threatening letters of copycats all over the world reawaked the interest in anthrax. The public was not only frightened by letters but also by all kinds of somehow suspicious looking substances (“white powders”). Hence hundreds of environmental samples had to be collected and analyzed for *B. anthracis*. This was one of the reasons why the Office of Waste, Water, Energy and Air, Biosafety unit of the canton of Zurich let establish a land register of possibly contaminated locations to get information on where in the canton *B. anthracis* could still occur “naturally”.

Remark: Data obtained by these investigations are for restricted use only due to legal regulations and protection of privacy. Therefore, only part of the data are given here as representative examples.

Methods

Sites to be taken into consideration are (former) industrial sites of companies with anthrax-relevant activities (handling potentially contaminated materials such as hides, fur, wool, hair, meat, or bone meal) and carcass disposal sites. Farms and pastures, where anthrax infections occurred, have further to be considered for the register. Relevant information to locate and assess the sites was obtained by investigation of historical sources.

Industrial sites: For information concerning industrial sites specific professional associations of the leather- and textile-industry were consulted. In addition, the old Zurich cantonal registry of commercial enterprises covering the period from 1842 to 1870 was screened. Furthermore, we looked in gazetteers for corresponding field and street names. Where necessary, additional information was obtained from communal offices and eldest people of a community.

Carcass burial sites: Prior to the ban to burry carcasses and the establishment of locations to collect carcasses, every political community had to designate an official carcass burial site (in German: “Wasenplatz”). Most of them were identified relatively accurately by surveying all 171 political communities of the canton of Zurich.

Infection sites: Information to specify sites where infections occurred was obtained from federal and cantonal offices (federal office of public health BAG, federal veterinary office BVET, cantonal health service and veterinarians) registers of epizootics (Kantonstierärzte Zürich 1878-1919; Bundesamt für Veterinärwesen 1900-2005) and from a report on an epidemic of anthrax (Pfisterer 1991). Precise addresses were not available anymore, only the affected communities.

Site assessment: For all sites an assessment of the probability of contamination and the actual infection risk was performed regarding the following criteria:

- origin of processed raw material (from regions with increased occurrence of anthrax)
- company or plant specifics (pit tannery, landfill)
- anthrax cases in the community (for burial sites)
- current state of the site (sealed/paved, open surface)

Sites

For legal reasons, community names have been anonymized.

Industrial sites

At most sites the ground has been paved and sealed. Thus, the risk of spore emission from the soil is rather low. Certain sites have been converted, buildings and installations demolished and new ones constructed. Table 1 gives an overview of the sites and their assessed risk.

Table 1: Overview of industrial sites in the canton of Zurich related to the occurrence of (animal) anthrax.
*assessment: ∅ = no risk, (!) = may be critical, ! = critical

community	industrial activity	comments	a*
5	tannery		∅
34	tannery, 1886-1926/29	next to the tannery peat was cut and backfilled with tannery waste after closing down, channels and pits were backfilled with excavated soil and rubble	∅
48	cord factory since 1872	1978-81 25 employees were infected by goat hair from Pakistan, only diagnosed in retrospect	∅
67	tannery		∅
85	tannery, 1866-1970 dumpsite, cold storage, tannery pit	serious fire in 1972, buildings not built up anymore pools of the wastewater treatment plant were backfilled without previous draining	!
89	tannery 1883-1930, pit tannery		(!)
111	horsehair spinnery	until 1936 anthrax occurred periodically on the surrounding farms	∅
134	tannery since 1883; pit drainage		∅
139	tannery		∅
151	horsehair spinnery	now lofts	∅
151	tannery		∅
161	tannery		∅
167	slaughterhouse since1938	before grassland former dumpsite adjacent	∅
167	gelatine factory since 1883		∅
167	tannery, 1833-1889 dyeing mill, 1845-1998		∅
171	company processing animal by-products (meat, hides, bones etc.)		∅
171	slaughterhouse since 1935 1913-1930 open pit	until 1907 grassland, 1907-09 construction of slaughterhouse	∅
171	carcass rendering plant until 1975	demolition	∅
171	dyeing mill dyeing mill / mill washer plant since 1883	wool from Australia, cape/South Africa, some from South America and Switzerland	∅

Carcass burial sites

Carcass burial sites of communities with registered anthrax cases have been classified as critical. But if these sites are overbuilt and the ground paved and sealed, it is assumed that there is no risk. Concerning the assessment, the following has to be considered: First, for most of the sites listed here it is not known since when they existed. Therefore, it is not clear, if the carcasses of the registered cases really have been buried there. Second, it is possible that there have been cases in a community but no information is available anymore. Corresponding carcass burial sites may be of risk although they have been classified here as nonhazardous. Table 2 gives an overview of the sites and their assessed risk. 28 sites are critical, 21 may be critical and 19 pose no risk.

Table 2: Overview carcass burial sites in the canton of Zurich.

*assessment: ∅ = no risk, (!) = may be critical, ! = critical

community	number of sites	in use until	comments	a*
5	1	1970		!
8	1	n.a.		∅
9	2	1940, 1970		∅
10	3	n.a.		!
11	2	1949, 1950		∅
13	1	n.a.		∅
15	2	1955		(!)
16	1	n.a.		!
18	1	n.a.		∅
19	1	n.a.		!
24	1	1960		!
25	3	1965		∅
26	1	1957		∅
32	1	1965	depression filled 25-30 years ago	!
33	1	1965		∅
35	1	n.a.		!
41	1	1960		(!)
47	3 (1 horse cemetry)	2 of them 1965		∅
48	1	1965		(!)
51	1	1950/60		(!)
52	1	n.a.		!
53	1	1970		(!)
54	1	n.a.		!
59	1	n.a.		!
60	1	1950/60		(!)
61	1	1971/72		!

community	number of sites	in use until	comments	a*
64	1	1978		∅
65	2	1965		(!)
66	1	1960		!
76	2	1965		!
80	1	1965		(!)
81	1	1980?		!
82	1	1965		(!)
83	1	1965		(!)
84	1	1970		∅
86	2	1960, 1975		!
89	1	1965		!
91	1	1974		!
93	3	2 of them 1956		!
96	1	1955		∅
102	1	1965		(!)
103	2	1965, 1970		∅
104	1	1980		(!)
110	1	1960		(!)
111	1	n.a.		!
112	1	n.a.		∅
118	1	1940/1960?		∅
119	1	n.a.		(!)
123	1	1960		!
125	2	1 of them 1965		(!)
129	1	never used		∅
130	1	n.a.		(!)
132	1	1967	now kindergarten, formerly used as general disposal site	(!)
134	1	1940		!
140	1	1965		!
144	1	1970		(!)
147	1	1932		∅
150	1	1955		!
151	1	1907		!
152	1	1940		!
153	3	2 of them 1930 1 of them 1970	now overbuilt	(!)
156	1	1970		!
160	1	1960		∅
161	1	1830?		!

community	number of sites	in use until	comments	a*
162	1	1965		∅
166	2	1 of them 1963		(!)
167	1	1928		!
168	4	1 of them 1965		(!)
171	1			∅

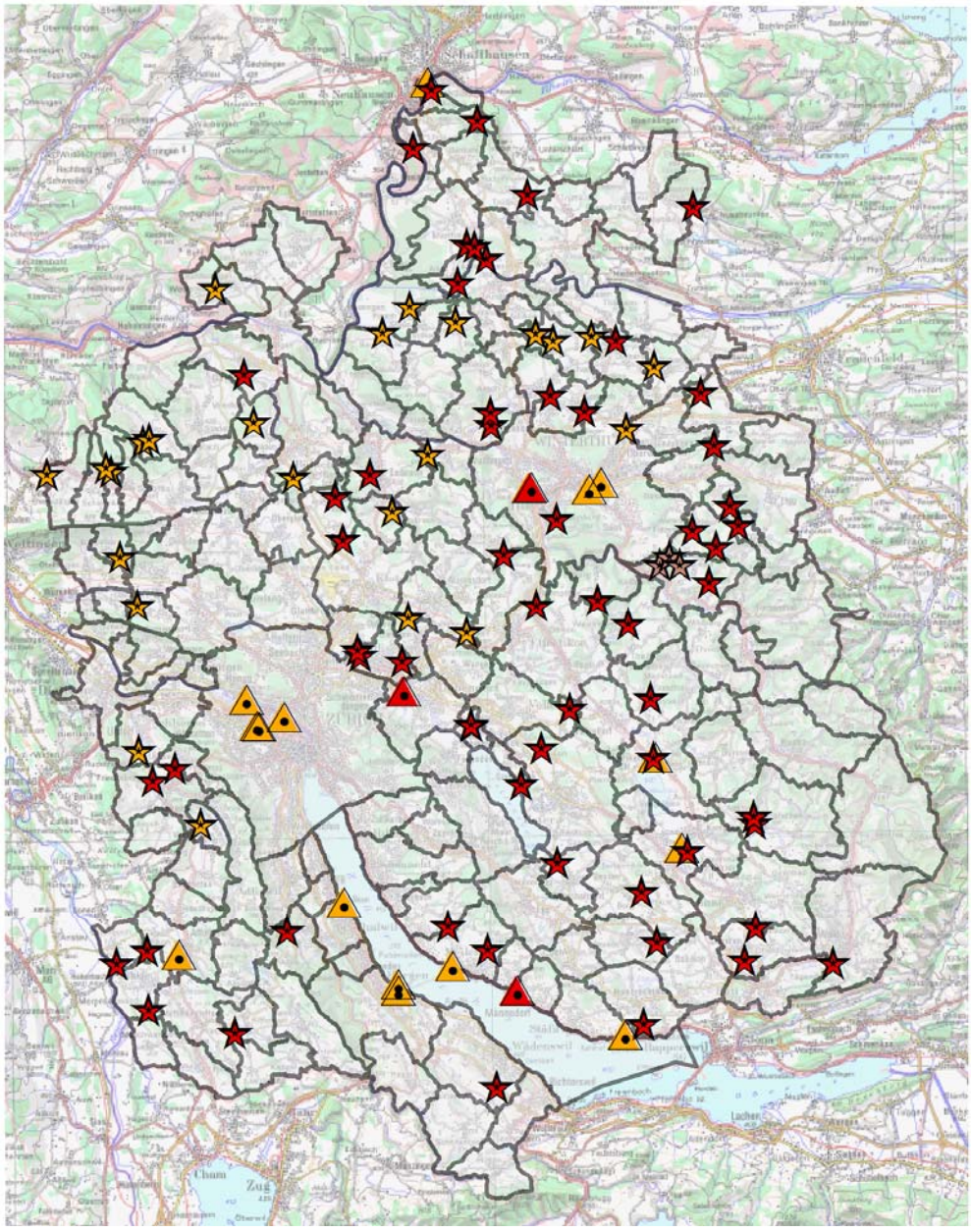

Fig. 1: Land register in a geographical information system, GIS. Overview of possibly anthrax-contaminated sites in the canton of Zurich. ★ are carcass burial sites, ▲ are industrial sites, red=critical, orange=no risk, brown=no information.

Geographical information system, GIS

All collected data were processed with ArcGIS and a land register in the form of a geographical information system was created. Carcass disposal sites are represented as point of mean coordinates. Industrial sites are either represented as point of mean coordinates or as polygon of the exact area, depending on the details of the available information. All sites are marked with a color coding for the risk of hazard: critical, no risk or no further information. When clicking on a carcass burial site the following information is shown: community, mean X- and Y-coordinate, last year of operation, and the occurrence of cases in the community. For industrial sites the following is shown: community, mean X- and Y-coordinate, type of industry, company name, and When clicking anywhere on the map the district, the community and the number of cases in the community between 1878 and 1919 are shown. The overview map is shown in Fig. 1.

Discussion / Conclusion

In the last centuries, animal anthrax definitely mattered in Switzerland and also in the canton of Zurich. Vaccination of animals and the prohibition of burying animals that died from anthrax as well as the disinfection of possibly contaminated raw material before import or processing led to a decrease of cases and virtual eradication of the disease. The history of anthrax in Switzerland is nowadays largely unknown to the public. Relevant chronicles and historical records are often missing. Data of human cases are almost not available anymore. Another problem posed the fear of loss of property and real estate value and expensive redevelopment, which embarrassed people to give information. For all these reasons it is rather difficult to unfurl the past and to find details for locations with possible occurrence of anthrax.

Most of the known industrial sites, where risky material has been processed, are now paved and sealed. Furthermore no infections occurred in the last years, neither in humans nor in animals. This leads to the conclusion, that the current situation in the canton of Zurich can be considered as nonhazardous. There is a certain possibility to find *B. anthracis* spores at one of the registered sites, but the probability might be very low. The emission of spores from the soil and a subsequent infection seams even more unlikely. Our findings and data assessments correspond also to a comprehensive study conducted in Germany (Kautzky 2001).

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Tier-Milzbrand in der Schweiz: Historische Fälle im Kanton Zürich zwischen 1878 und 1919

Andrea Brandes und Helmut Brandl (Zürich)

Zusammenfassung

Historische Aufzeichnungen über Milzbrandfälle bei Tieren im Kanton Zürich zwischen 1878 und 1919 wurden auf der Ebene von politischen Gemeinden analysiert, wobei das Auftreten und die Anzahl von Fällen, die erkrankten Tierarten und die Anzahl betroffener Gemeinden untersucht wurden. Die Daten wurden sowohl mit den industriellen Aktivitäten (Gerben, Woll- und Rosshaarverarbeitung) in den Gemeinden als auch mit den vorherrschenden meteorologischen Bedingungen korreliert. Insgesamt wurden 675 Milzbrandfälle bei Tieren in 131 von 171 Zürcher Gemeinden verzeichnet, wobei mehrheitlich Rinder betroffen waren. Das Auftreten der Fälle korrelierte mit industriellen Aktivitäten in der jeweiligen Gemeinde. Frühere industrielle Aktivitäten (d. h. Unternehmen, die potenziell kontaminiertes Material, wie beispielsweise Häute, Felle, Wolle, Haar, Fleisch oder Knochenmehl verarbeiteten) zeigten einen positiven Zusammenhang zwischen dem Auftreten von Milzbrandfällen in einer Gemeinde und dem Vorhandensein bestimmter lokaler Betriebe. Der Ein uss von wollverarbeitenden Betrieben *(P=0.004)* und Gerbereien *(P=0.032)* erwies sich als erheblich, während rosshaarverarbeitende Betriebe keinen Ein- uss aufwiesen. In denjenigen Gemeinden, welche die grösste Anzahl Fälle verzeichneten, waren Gerbereien oder wollverarbeitende Betriebe ansässig.

Historical cases of animal anthrax in Switzerland: Incidents in the canton of Zurich between 1878 and 1919

Anthrax is a well-known infectious disease occurring in wild animals and livestock. The causing agent is the spore-forming, Gram-positive bacterium *Bacillus anthracis.* Anthrax might occur in horses, cattle, sheep, goats, pigs, buffaloes, camels, antelopes, other herbivores, and even in ostriches and elephants. It has been assumed that anthrax incidents are related to climatic as well as particular soil conditions because cases occurred always after long periods of rain or oodings. Additionally, carcass disposal sites have been identified as possible infection source. Historical records reporting cases of animal anthrax in the canton of Zurich between 1878 and 1919 were analysed on the level of political communities regarding occurrence and number of cases, animals affected, and number of municipalities affected. Data were correlated with industrial activities (tanning, wool and horse hair processing) in a community and to the prevailing meteorological conditions. A total of 675 cases of animal anthrax have been recorded showing a maximum in 1894. Most of cases involved cattle. Only 6 goats (0.9%), 16 pigs (2.3%) and 21 horses (3.1%) were affected. Cases occurred in 131 of the 171 communities over the 41 years of records. Occurrence correlated with industrial activities in a community. The investigation of relevant industrial activities (companies handling potentially contaminated materials, such as hides, fur, wool, hair, meat, or bone meal) showed that there is a correlation between the occurrence of cases in a community and local companies. The in uence of wool processing companies (*P=0.004*) and tanneries (*P=0.032*) is significant whereas horse hair processing showed no significance. In the communities reporting the highest numbers of cases, tanneries or wool processing industries were localized. However, a statistical relationship between the number of cases reported and meteorological data (rainfall, mean temperature) was not found.

Schlagwörter: Anthrax – *Bacillus anthracis* – Gerbereien – Milzbrand – Rosshaarverarbeitung – Wollverarbeitung

1 EINLEITUNG

Milzbrand ist eine bekannte Infektionskrankheit, die sowohl bei Wildtieren als auch bei Nutztieren wie Pferden, Rindern, Schafen, Ziegen, Schweinen, Büffeln, Kamelen, Antilopen und sogar bei Straussen und Elefanten vorkommt (DRAGON et al., 2005; GATES et al., 1995; HUGH-JONES und DE VOS, 2002; WATSON und KEIR, 1994). Der Erreger ist das sporenbildende grampositive Bakterium *Bacillus anthracis* (DRAGON und RENNIE, 1995).

B. anthracis ist ein weit verbreitetes Bodenbakterium und kommt weltweit vor. In einigen Regionen ist die Krankheit endemisch (Asien, Zentralafrika, südlicher Teil der USA sowie Südamerika), in den meisten anderen Erdteilen kommt sie sporadisch vor. In Europa ist *B. anthracis* in Spanien, Griechenland, Albanien und Mazedonien endemisch. Die Niederlande, Belgien, Luxemburg, Österreich, die Tschechische Republik, die Slowakei, Lettland, Estland, Skandinavien, Dänemark, Irland und Island scheinen von Milzbrand nicht betroffen zu sein. Aktuelle Informationen sind unter http://www.vetmed.lsu.edu/whocc/mp_world.htm zu finden.

Es gibt nur wenige historische Aufzeichnungen von Milzbrand bei Tieren. Berichte über Ausbrüche bei Nutztieren in den Vereinigten Staaten von Amerika reichen bis ins Jahr 1834 zurück (STEIN, 1953), während in Deutschland Milzbrand bei Tieren Ende des 19. und Anfang des 20. Jahrhunderts weit verbreitet war (ZEPEZAUER und BOCKLISCH, 1980). Zwischen 1914 und 1929 wurden in Deutschland 20 279 Fälle verzeichnet, mit einem Maximum von 6847 toten Tieren im Jahr 1914 (STANDFUSS, 1958). Die Mortalität der infizierten Tiere lag zwischen 88% und 97%. Das vermehrte Vorkommen von Milzbrand wird sowohl mit der Industrialisierung als auch mit dem gesteigerten Import von Tierprodukten, insbesondere getrockneten Häuten, in Zusammenhang gebracht (SCHIESS, 1997). Ausserdem wird angenommen, dass das Vorkommen von Milzbrand mit klimatischen Bedingungen sowie bestimmten Bodenbedingungen zusammenhängt, da Fälle von Erkrankungen stets nach langen Regenperioden oder Überschwemmungen auftreten (PEPPER und GENTRY, 2002; SCHIESS, 1997; ZEPEZAUER, 1980). Andere historische Quellen schreiben das Auftreten von Tier-Milzbrand hohen Temperaturen zu. So berichtet WITTA (WITTA, 1826) über einen Ausbruch im Jahre 1821 im Kanton St. Gallen, der «anfangs Brachmonath» [Juni] begann und «im Heumonath [Juli] endigte», verursacht «durch die grosse Hitze dieses Sommers». Ebenso trat Milzbrand im September 1822 in den Kantonen Schwyz und Zug auf. «Als die Ursache hiervon wurde die grosse Hitze des Sommers angegeben» (RICKENBACH und SCHLUMPF, 1826).

Des Weiteren wurden Wasenplätze als mögliche Infektionsquellen identifiziert (SCHIESS, 1997; ZEPEZAUER, 1980). Wasenplätze (von Wasen: feuchter Rasen) sind ausgesonderte Standorte, wo Tierkadaver entsorgt bzw. vergraben werden. Daneben kam es auch zur «Entsorgung» von Tierkadavern in Sümpfen. Der Wortstamm «Cheib» (berndeutsch für «Aas» oder alemannisch für «Tiere, die vergraben wurden») deutet auf solche Lokalitäten hin. Während des Ersten Weltkriegs wurden weniger Tierkadaver im Boden verscharrt als früher; gleichzeitig nahm die Zahl der Milzbrandfälle in Europa stark ab (STANDFUSS, 1958).

In der Schweiz traten die ersten dokumentierten Milzbrandfälle bei Tieren zwischen 1818 und 1820 auf dem Bauernhof Schwängi bei Langenbruck (BL) südwestlich von Basel auf (SACKMANN, 1994). Der letzte grosse Ausbruch ereignete sich 1985 im Kanton Graubünden, wo elf Kühe und Rinder sowie zwei Ziegen starben (KUONI und ZINDEL, 1986). Seit 1997 sind in der Schweiz keine weiteren Fälle von Milzbrand verzeichnet worden (MISSURA, 2001).

Basierend auf historischen Aufzeichnungen wird in diesem Beitrag über alle Milzbrandfälle bei Tieren im Kanton Zürich zwischen 1878 und 1919 berichtet. Diese Daten werden mit damaligen industriellen Aktivitäten, die vermutlich mit Milzbrand in Zusammenhang stehen (wie z. B. Gerben oder Wollverarbeitung), sowie mit den meteorologischen Daten aus diesem Zeitraum korreliert.

2 VERARBEITUNG VON GRUNDLAGENDATEN

Die historischen Aufzeichnungen stammen vom Kantonalen Veterinäramt Zürich und wurden vorwiegend dem kantonalen Tierseuchenregister aus dem Zeitraum zwischen 1878 und 1919 entnommen. Die Verfügbarkeit dieser Daten ist sehr aussergewöhnlich, da die Aufbewahrungsp icht für solche Akten offiziell nur 10 Jahre beträgt. Die einzelnen

Fälle wurden nach politischen Gemeinden analysiert und ausgewertet. Nachfolgend wird ein Fall als ein Ausbruch an einem Ort zu einer bestimmten Zeit definiert, wobei jeweils pro Fall ein oder mehrere Tiere betroffen sein konnten. Total gibt es im Kanton Zürich 171 politische Gemeinden auf einer Fläche von 1729 km^2. Angaben zu relevanten früheren industriellen Aktivitäten in allen Gemeinden (namentlich das Vorhandensein von Schlachthäusern, Gerbereien, fellverarbeitenden Betrieben, Webereien und Spinnereien) sind dem Handelsregister des Kantons Zürich entnommen und decken die Periode von 1842 bis 1870 ab. Wo nötig wurden zusätzliche Informationen von den Gemeindeverwaltungen bezogen. Die meteorologischen Daten für den Zeitraum von 1878 bis 1919 (monatliche Durchschnittstemperatur, monatliche Gesamtniederschlagsmenge) wurden von MeteoSchweiz zur Verfügung gestellt (BEGERT et al., 2005). Statistische Analysen wurden mit dem Open Source Software Package R (R DEVELOPMENT CORE TEAM, 2004) durchgeführt. Die Anzahl Milzbrandfälle bei Tieren wurde als Funktion der Anzahl Gerbereien, wollverarbeitenden Betrieben und rosshaarverarbeitenden Betriebe analysiert. Die Signifikanz wurde mittels Devianzanalyse mit dem Chi-Quadrat-Test untersucht.

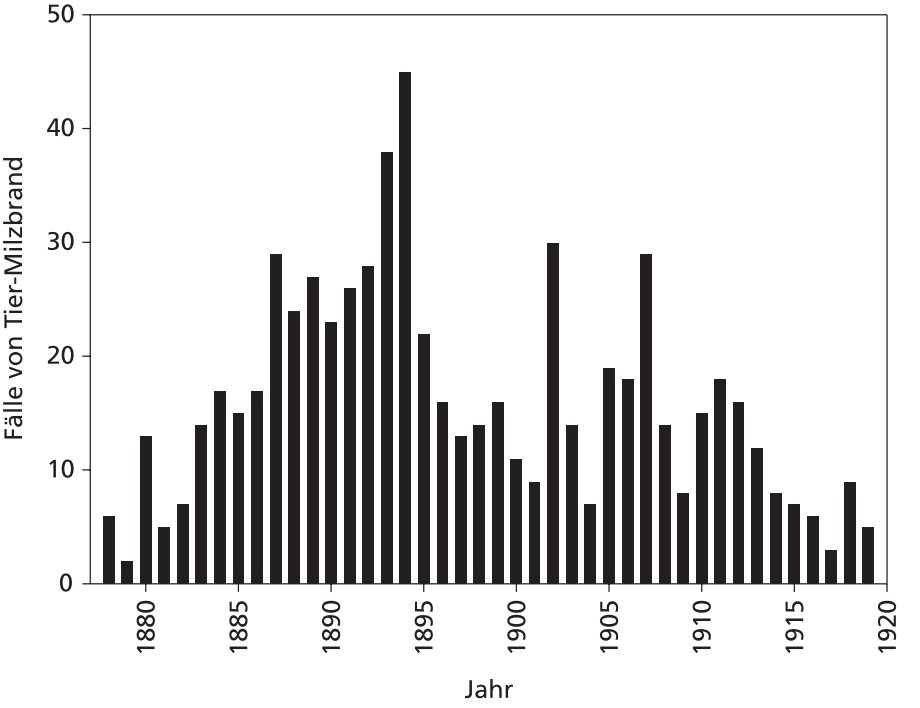

Abb. 1. Jährliches Auftreten von Tier-Milzbrand im Kanton Zürich zwischen 1878 und 1919. Die Daten wurden dem kantonalen Tierseuchenregister entnommen.

Fig. 1. Animal anthrax in the canton of Zurich (Switzerland) between 1878 and 1919 reported as the number of cases per year. Data are based on the Zurich cantonal register of epizootics.

3 HISTORISCHE FÄLLE VON MILZBRAND BEI TIEREN IM KANTON ZÜRICH

Zwischen 1878 und 1919 wurden im Kantonalen Tierseuchenregister insgesamt 675 Milzbrandfälle bei Tieren verzeichnet, wobei 1894 die maximale Anzahl Fälle (45) registriert wurde (Abb. 1). Bei den meisten der 675 Fälle handelte es sich um Rinder, während nur 6 Ziegen (0,9%), 16 Schweine (2,3%) und 21 Pferde (3,1%) betroffen waren. Die Zahl der dokumentierten Fälle nahm zwischen 1880 und 1894 kontinuierlich zu und ging danach abgesehen von zwei weiteren Höchstwerten in den Jahren 1902 (30 Fälle) und 1907 (29 Fälle) wieder zurück. Es ist schwierig, daraus eine kausale Beziehung abzuleiten, doch die zunehmende Industrialisierung (einschliesslich des erhöhten Imports von Fellen und Häuten sowie der vermehrten Verarbeitung von Tierprodukten) während der Zeit um den Wechsel vom 19. ins 20. Jahrhundert könnte zu dieser Entwicklung beigetragen haben. Nach 1894 ist eine kontinuierliche Abnahme von Milzbrand bei Tieren feststellbar.

Während der im Rahmen dieser Studie untersuchten Zeitdauer von 41 Jahren wurde in 131 von den 171 Gemeinden im Kanton Zürich mindestens ein Fall von Milzbrand bei Tieren festgestellt. In 58% der Gemeinden traten in diesem Zeitraum nur wenige Fälle auf (1 bis 5), während nur in 5 Gemeinden (3%) mehr als 20 Fälle verzeichnet wurden (Abb. 2). In ungefähr einem Viertel der politischen Gemeinden ereigneten sich keine Milzbrandfälle. Die Untersuchung von relevanten industriellen Aktivitäten (Gewerbe, die potenziell kontaminiertes Material wie beispielsweise Häute, Felle, Wolle, Haar, Fleisch oder Knochenmehl verarbeiteten) ergaben, dass ein positiver Zusammenhang zwischen dem Auftreten von Milzbrand in einer Gemeinde und dem Vorhandensein bestimmter lokaler Betriebe besteht

(Tab. 1). Der Ein uss von wollverarbeitenden Betrieben *(P=0.004)* und Gerbereien *(P=0.032)* erwies sich als erheblich, während rosshaarverarbeitende Betriebe keinen Ein uss hatten *(P=0.914)*. In den Gemeinden mit der grössten Anzahl Fälle waren Gerbereien oder wollverarbeitende Betriebe ansässig.

Da bestimmte Wetterbedingungen (insbesondere Regenfälle, Überschwemmungen, Hitzeperioden) die Häufigkeit von Milzbrandfällen bei Tieren erhöhen könnten, wurden die historischen Fälle mit den meteorologischen Daten aus den Jahren zwischen 1878 und 1919 verglichen. Die Analyse ergab, dass kein Zusammenhang zwischen der Zahl der dokumentierten Milzbrandfälle und dem monatlichen Gesamtniederschlag (Abb. 3) oder der monatlichen Durchschnittstemperatur besteht (ohne Abbildung). Ausserdem wurden die monatlichen Niederschlagsmengen einen Monat VOR dem Auftreten der Milzbrandfälle mit den Erkrankungen bei Tieren korreliert, um festzustellen, ob vorhergehende Witterungsbedingungen einen Ein uss auf das Vorkommen von Milzbrandfällen hatten. Es war jedoch auch diesbezüglich keine signifikante Korrelation festzustellen.

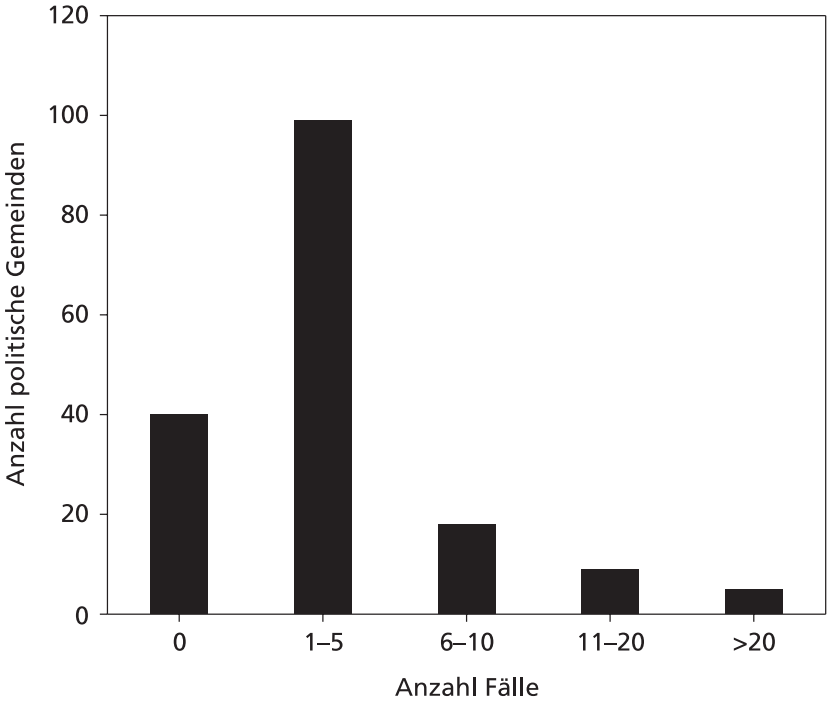

Abb. 2. Fälle von Tier-Milzbrand in den 171 Gemeinden des Kantons Zürich zwischen 1878 und 1919.

Fig. 2. Cases of animal anthrax in the 171 communities of the canton of Zurich (Switzerland) between 1878 and 1919.

Tab. 1. Korrelation zwischen dem Auftreten von Tier-Milzbrand in politischen Gemeinden und lokalen relevanten Gewerbebetrieben.

Tab. 1. Correlation between the occurrence of cases in a political community and local industries.

	Freiheitsgrad	Devianz	P(>|Chi|)
Gerberei	1	4,583	0,032
Wollverarbeitung	1	8,481	0,004
Rosshaarverarbeitung	1	0,012	0,914
Residuen	167	181,981	–

4 SCHLUSSFOLGERUNGEN

Historische Aufzeichnungen belegen, dass Milzbrand im Kanton Zürich weit verbreitet war, wobei im Jahr 1894 eine Höchstzahl von Fällen verzeichnet wurde, gefolgt von einem kontinuierlichen Rückgang. Die statistische Analyse ergab, dass ein signifikanter Zusammenhang zwischen dem Auftreten von Milzbrandfällen einerseits und dem Vorhandensein von wollverarbeitenden Betrieben und Gerbereien andererseits besteht. Eine Korrelation von rosshaarverarbeitenden Betrieben mit dem Vorkommen von Milzbrand war jedoch nicht feststellbar. Die Hauptursache für den Rückgang von Milzbrandfällen bei Tieren in den industrialisierten Ländern dürfte das Verbot gewesen sein, infizierte Tierkadaver zu verscharren. Diese zuvor weit verbreitete Praxis stellte sicherlich ein beträchtliches Risiko für die Verbreitung dar, was sich im vermehrten Auftreten von Milzbrand in Gemeinden mit wollverarbeitenden Betrieben und Gerbereien widerspiegeln könnte, wo Kadaver direkt verscharrt oder auf nahe gelegenen Deponien entsorgt wurden (SCHIESS, 1997).

Die Industrialisierung und der damit einhergehende Handel begünstigten die weltweite Verbreitung von Milzbrand (STANDFUSS, 1958). Das Impfen von Tieren und die Sterilisierung von möglicherweise kontaminiertem Rohmaterial vor dem Import führten zu einem Rückgang von Milzbrandfällen. Da die Sporen von *B. anthracis* äusserst resistent sind, kann das Bakterium über Jahre oder gar Jahrzehnte überleben und sporadisch Infektionen auslösen. Mehrere Studien legen dar, dass Infektionen oft

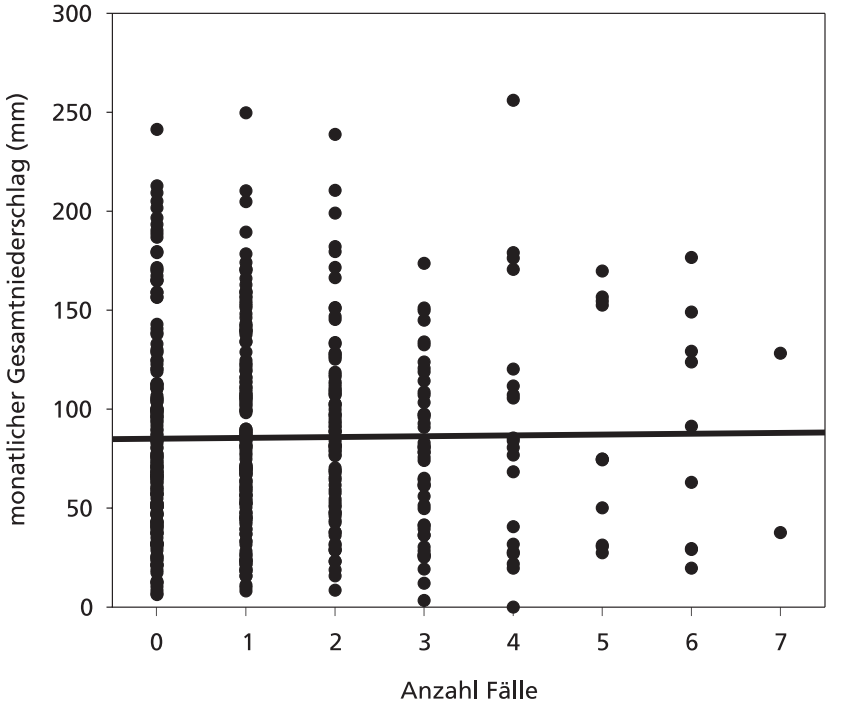

Abb. 3. Korrelation des monatlichen Gesamtniederschlags mit dem Auftreten von Tier-Milzbrand (total 675 Fälle). Die Linie stellt die Regressionsgerade y=0.42x + 85.0 (r^2=0.000153) dar.

Fig. 3. Monthly number of anthrax cases (total 675 cases) in relation to total monthly rainfall between 1878 and 1919. The solid line represents the linear regression y=0.42x + 85.0 (r^2=0.000153).

nach grossen Regenfällen in der warmen Jahreszeit auftreten, vermutlich weil die Bacillus-Sporen dann aus dem Boden geschwemmt werden und sich in Senken ansammeln können (LINDEQUE und TURNBULL, 1994; PEPPER und GENTRY, 2002; ZEPEZAUER, 1980). Im Gegensatz zu anderen Studien konnte keine Korrelation zwischen der Zahl der Milzbrandfälle und den meteorologischen Daten, d. h. Niederschlagsmenge oder Durchschnittstemperatur, festgestellt werden. Allerdings zeigen die verwendeten meteorologischen Daten nur monatliche Durchschnittswerte für den Kanton Zürich auf und nicht die spezifischen Tageswerte für jede einzelne Gemeinde im Kanton; sie könnten somit zu wenig genau sein.

5 VERDANKUNGEN

Unser spezieller Dank gilt den Studentinnen und Studenten des Studiengangs «Übersetzen» der Zürcher Hochschule Winterthur, welche im Rahmen einer Projektarbeit den Originaltext aus dem Englischen übersetzt haben. Die Arbeit wurde durch das Amt für Abfall, Wasser, Energie und Luft (AWEL) des Kantons Zürich unterstützt. Wir danken dem Veterinäramt des Kantons Zürich, welches uns Einsicht in das Tierseuchenregister gewährt hat. Ebenso danken wir dem Verband schweizerischer Gerbereien (VSG), dem Textilverband Schweiz (TVS), Bruno Aemissegger (Eskimo Textil AG), Ernst Nef, Romain Rueff und Hanspeter Zingg und der Firma Matousek, Baumann & Niggli AG für die Überlassung von relevanten Informationen.

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Milzbrand im Kanton Zürich zwischen 1878 und 2005

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Zusammenfassung

Historische Aufzeichnungen über Milzbrandfälle bei Tieren im Kanton Zürich zwischen 1878 und 2005 wurden auf der Ebene von politischen Gemeinden analysiert, wobei das Auftreten und die Anzahl von Fällen, die erkrankten Tierarten und die Anzahl betroffener Gemeinden untersucht wurden. Die Daten wurden sowohl mit den industriellen Aktivitäten (Gerben, Woll- und Rosshaarverarbeitung) in den Gemeinden als auch mit den vorherrschenden meteorologischen Bedingungen korreliert. In der Untersuchungsperiode wurden insgesamt 830 Milzbrandfälle bei Tieren in 140 von 171 Zürcher Gemeinden verzeichnet, wobei mehrheitlich Rinder betroffen waren. Das Auftreten der Fälle korrelierte mit industriellen Aktivitäten in der jeweiligen Gemeinde. Ein positiver Zusammenhang zwischen dem Auftreten von Milzbrandfällen in einer Gemeinde und dem Vorhandensein lokaler Unternehmen, die potenziell kontaminiertes Material wie Häute, Felle, Wolle, Haar, Fleisch oder Knochenmehl verarbeiteten, konnte gezeigt werden. Der Einfluss von wollverarbeitenden Betrieben *(P = 0.004)* und Gerbereien *(P = 0.032)* erwies sich als erheblich, während rosshaarverarbeitende Betriebe keinen Einfluss hatten. Zwischen dem Auftreten von Milzbrandfällen und meteorologischen Bedingungen (Niederschlag, Temperatur) gab es keinen statistisch signifikanten Zusammenhang.

Schlüsselwörter: Anthrax, *Bacillus anthracis,* Gerbereien, Milzbrand, Rosshaarverarbeitung, Wollverarbeitung

Anthrax in the canton of Zurich between 1878 and 2005

Historical records reporting cases of animal anthrax in the canton of Zurich between 1878 and 2005 were analysed on the level of political communities regarding occurrence and number of cases, animals affected, and number of communities affected. Data were correlated with industrial activities (tanning, wool and horse hair processing) in a community and to the prevailing meteorological conditions. A total of 830 cases of animal anthrax has been recorded in 140 of 171 communities. Occurrence correlated with industrial activities in a community such as companies handling potentially contaminated materials (hides, fur, wool, hair, meat, or bone meal). The influence of wool processing companies *(P = 0.004)* and tanneries *(P = 0.032)* was significant whereas horse hair processing had no effect. However, a statistical relationship between the number of cases reported and meteorological data (rainfall, mean temperature) was not found.

Keywords: anthrax, *Bacillus anthracis,* tanneries, wool processing

Einleitung

Milzbrand ist eine bekannte Infektionskrankheit, die sowohl bei Wildtieren als auch bei Nutztieren wie Pferden, Rindern, Schafen, Ziegen, Schweinen, Büffeln, Kamelen, Antilopen und sogar bei Straussen und Elefanten vorkommt (Watson und Keir, 1994; Gates et al., 1995; Hugh-Jones und de Vos, 2002; Dragon et al., 2005). Der Erreger ist das sporenbildende, grampositive Bakterium *Bacillus anthracis* (Dragon und Rennie, 1995).

B. anthracis ist ein weit verbreitetes Bodenbakterium und kommt weltweit vor. In einigen Regionen ist die Krankheit endemisch (Asien, Zentralafrika, südlicher Teil der USA sowie Südamerika), in den meisten anderen Erdteilen kommt sie sporadisch vor. In Europa ist *B. anthracis* in Spanien, Griechenland, Albanien und Mazedonien endemisch. Die Niederlande, Belgien, Luxemburg, Österreich, die Tschechische Republik, die Slowakei, Lettland, Estland, Skandinavien, Dänemark, Irland und Island scheinen von Milzbrand nicht betroffen zu sein. Aktuelle Informationen sind unter http://www.vetmed.lsu.edu/whocc/mp_world.htm zu finden.

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In Deutschland war Milzbrand Ende des 19. und Anfang des 20. Jahrhunderts weit verbreitet (Zepezauer und Bocklisch, 1980). Im Zeitraum von 1893 bis 1899 erkrankten 29'686 Tiere an Milzbrand (Felix, 1905), und zwischen 1914 und 1929 wurden 20'279 Fälle verzeichnet, mit einem Maximum von 6847 toten Tieren im Jahr 1914 (Standfuss, 1958). Die Mortalität der infizierten Tiere lag zwischen 88% und 97%. Das vermehrte Vorkommen von Milzbrand wird sowohl der Industrialisierung als auch dem gesteigerten Import von Tierprodukten, insbesondere getrockneten Häuten, zugeschrieben (Schiess, 1997). Oft traten gehäuft Fälle in der Umgebung von Rosshaar- und Kammgarnspinnereien auf (Silberschmidt, 1896). Den Zusammenhang zwischen Gewerbe und Milzbrand zeigt auch die vom Bundesrat 1887 erlassene erste sogenannte Giftliste zum ersten Fabrikgesetz der Schweiz, in der Milzbrand zusammen mit 13 anderen Komponenten wie z.B. Salzsäure oder Nitroglycerin aufgeführt war (Wegmann, 1934). Diese Liste wurde 1916 erweitert, wobei aber Milzbrand (zusammen mit Pocken und Rotz) gestrichen wurde (Zollinger, 1930).

Neben dem direkten Zusammenhang mit industriellen Aktivitäten wurde vermutet, dass das Vorkommen von Milzbrand mit spezifischen klimatischen Bedingungen zusammenhängt, da Fälle von Erkrankungen stets nach langen Regenperioden oder Überschwemmungen auftraten (Zepezauer, 1980; Schiess, 1997; Pepper und Gentry, 2002). Andere historische Quellen schreiben das Auftreten von Tier-Milzbrand hohen Temperaturen zu. So wird über einen Ausbruch im Jahre 1821 im Kanton St. Gallen berichtet, der «anfangs Brachmonath» [Juni] begann und «im Heumonath [Juli] endigte», verursacht «durch die grosse Hitze dieses Sommers» (Witta, 1926). Ebenso trat Milzbrand im September 1822 in den Kantonen Schwyz und Zug auf. «Als die Ursache hiervon wurde die grosse Hitze des Sommers angegeben» (Rickenbach und Schlumpf, 1826).

Des Weiteren wurden Wasenplätze als mögliche Infektionsquellen identifiziert (Zepezauer, 1980; Schiess, 1997). Wasenplätze (von Wasen: feuchter Rasen) sind ausgesonderte Standorte, wo Tierkadaver entsorgt bzw. vergraben werden. Daneben kam es auch zur „Entsorgung" von Tierkadavern in Sümpfen. Der Wortstamm „Cheib" (berndeutsch für „Aas" oder alemannisch für „Tiere, die vergraben wurden") deutet auf solche Lokalitäten hin. Während des Ersten Weltkriegs wurden weniger Tierkadaver im Boden verscharrt als früher; gleichzeitig nahm die Zahl der Milzbrandfälle in Europa stark ab (Standfuss, 1958).

In der Schweiz traten die ersten dokumentierten Milzbrandfälle bei Tieren zwischen 1818 und 1820 auf dem Bauernhof Schwängi bei Langenbruck (BL) auf (Sackmann, 1994). Der letzte grosse Ausbruch ereignete sich 1985 im Kanton Graubünden, wo elf Kühe und Rinder sowie zwei Ziegen starben (Kuoni und Zindel, 1986). Seit 1997 sind in der Schweiz keine weiteren Fälle von Milzbrand aufgetreten (Missura, 2001; Bundesamt für Veterinärwesen, 1900-2005).

Material und Methoden

Basierend auf den kantonalen Aufzeichnungen (Tierseuchenregister) von 1878 bis 1919 umfassen die Untersuchungen nur Milzbrandfälle bei Tieren im Kanton Zürich. Eine Erweiterung der handschriftlichen Aufzeichnungen erfolgte durch die Statistik des Bundesamtes für Veterinärwesen bis ins Jahr 2005. Die historischen Daten von 1878 bis 1919 wurden mit damaligen industriellen Aktivitäten, die vermutlich mit Milzbrand in Verbindung standen (Gerben, Woll- oder Rosshaarverarbeitung), verglichen, um einen allfälligen Zusammenhang zwischen Gewerbe und dem Auftreten von Milzbrand aufzuzeigen. Alle Daten wurden meteorologischen Daten (Niederschlag, Temperatur) für diese Zeitspanne bis zum Auftreten des letzten Falles (1969) gegenübergestellt, um Verbindungen zwischen Witterungsverhältnissen und dem Auftreten von Milzbrand zu erkennen. Uns sind keine Arbeiten bekannt, welche diese Zusammenhänge statistisch untersucht haben.

Die Verfügbarkeit dieser Daten ist aussergewöhnlich, da die Aufbewahrungspflicht für solche Akten offiziell nur 10 Jahre beträgt. Die einzelnen Fälle wurden nach politischen Gemeinden analysiert und ausgewertet. Nachfolgend wird ein Fall als ein Ausbruch an einem Ort zu einer bestimmten Zeit definiert, wobei jeweils pro Fall ein oder mehrere Tiere betroffen sein konnten. Total gibt es im Kanton Zürich 171 politische Gemeinden, zusammengefasst in 12 Bezirke, auf einer Fläche von 1729 km^2. Angaben zu relevanten früheren industriellen Aktivitäten in allen Gemeinden (namentlich das Vorhandensein von Schlachthäusern, Gerbereien, fellverarbeitenden Betrieben, Webereien und Spinnereien) sind dem Handelsregister des Kantons Zürich entnommen und decken die Periode von 1842 bis 1870 ab. Wo nötig, wurden zusätzliche Informationen von den Gemeindeverwaltungen bezogen. Die meteorologischen Daten für den Zeitraum von 1878 bis zum Auftreten des letzten Falles im Jahr 1969 (monatliche Durchschnittstemperatur, monatliche Gesamtniederschlagsmenge im Kanton Zürich) wurden von MeteoSchweiz zur Verfügung gestellt (Begert et al., 2005). Statistische Analysen wurden mit dem Open Source Software Package R (R Development Core Team, 2004) durchgeführt. Die Anzahl Milzbrandfälle bei Tieren wurde als Funktion der Anzahl Gerbereien, wollverarbeitender Betriebe und rosshaarverarbeitender Betriebe analysiert. Die Signifikanz wurde mittels Devianzanalyse mit dem Chi-Quadrat-Test untersucht.

Ergebnisse

Zwischen 1878 und 2005 wurden im Kantonalen Tierseuchenregister insgesamt 830 Milzbrandfälle bei Tieren verzeichnet, wobei 1894 die maximale Anzahl Fälle (45) registriert wurde (Abb. 1). Bei den meisten der 830 Fälle handelte es sich um Rinder (91.8%), während nur 8 Ziegen (1.0%), 39 Schweine (4.6%) und 22 Pferde (2.6%) betroffen waren. Die Zahl der dokumentierten Fälle nahm zwischen 1880 und 1894 kontinuierlich zu und ging danach abgesehen von zwei weiteren Höchstwerten in den Jahren 1902 (30 Fälle) und 1907 (29 Fälle) wieder zurück. Während der im Rahmen dieser Studie untersuchten Zeitdauer von 127 Jahren wurde in 140 von den 171 Gemeinden im Kanton Zürich mindestens ein Fall von Milzbrand bei Tieren festgestellt (Abb. 2). In 56% der Gemeinden traten in diesem Zeitraum nur wenige Fälle auf (1 bis 5). In 13% der Gemeinden waren 6 bis 10 Fälle zu verzeichnen, in 9% 11 bis 20. Nur in 6 Gemeinden (4%) wurden mehr als 20 Fälle verzeichnet. In 18% der politischen Gemeinden ereigneten sich keine Milzbrandfälle.

Für die Untersuchung relevanter industrieller Aktivitäten, d.h. Gewerbe, die potenziell kontaminiertes Material wie Häute, Felle, Wolle, Haar, Fleisch oder Knochenmehl verarbeiteten, wurden aufgrund der eingeschränkten Verfügbarkeit von Daten nur die historischen Fälle bis 1919 miteinbezogen. Es zeigte sich, das ein positiver Zusammenhang zwischen dem Auftreten von Milzbrand in einer Gemeinde und dem Vorhandensein bestimmter lokaler Betriebe besteht (Tab. 1). Der Einfluss von wollverarbeitenden Betrieben *(P = 0.004)* und Gerbereien *(P = 0.032)* erwies sich als erheblich, während rosshaarverarbeitende Betriebe keinen Einfluss hatten *(P = 0.914)*. In den Gemeinden mit der grössten Anzahl Fälle waren Gerbereien und/oder woll- und/oder rosshaarverarbeitende

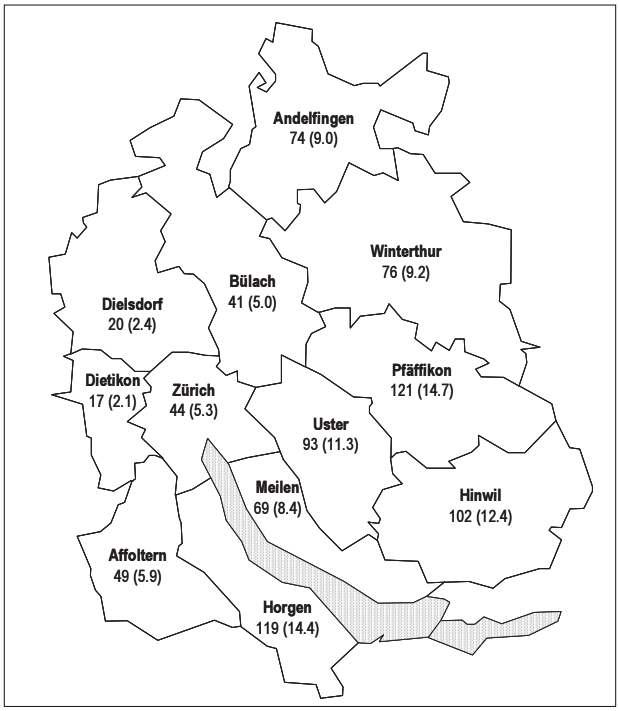

Abbildung 2: Fälle von Tier-Milzbrand in den 171 Gemeinden des Kantons Zürich zwischen 1878 und 2005 aufgeführt pro Bezirk als absolute Zahl und in Prozent (in Klammer).

Tab. 1. Zusammenhang zwischen dem Auftreten von Tier-Milzbrand zwischen 1878 und 1919 in politischen Gemeinden des Kantons Zürich und lokalen relevanten Gewerbebetrieben. Die Signifikanz wurde mittels Devianzanalyse (Chi-Quadrat-Test) untersucht.

	Freiheitsgrad	Devianz	P(>|Chi|)
Gerberei	1	4.583	0.032
Wollverarbeitung	1	8.481	0.004
Rosshaar-verarbeitung	1	0.012	0.914
Residuen	167	181.981	---

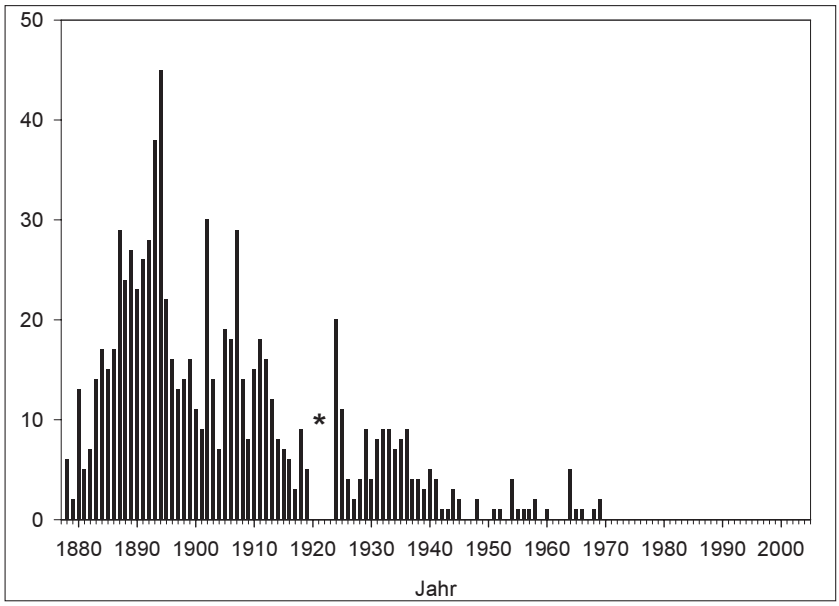

Abbildung 1: Jährliches Auftreten von Tier-Milzbrand im Kanton Zürich zwischen 1878 und 2005. Die Daten wurden dem kantonalen Tierseuchenregister und den Mitteilungen des Bundesamtes für Veterinärwesen entnommen. Für die Zeit von 1920 bis 1923 konnten keine Daten gefunden werden (*).

Betriebe ansässig. Die Fälle im Kanton Zürich zwischen 1878 und 1919 umfassen etwa 81% aller erfassten Fälle bis 2005.

Das Vorkommen von Milzbrand wurde mit meteorologischen Daten aus den Jahren zwischen 1878 und 1969, als der letzte Fall im Kanton auftrat, verglichen. Die Analyse ergab keinen Zusammenhang zwischen der Zahl der dokumentierten Milzbrandfälle und dem monatlichen Gesamtniederschlag oder der monatlichen Durchschnittstemperatur im Kanton Zürich (Abb. 3). Ein Vergleich des Auftretens von Milzbranderkrankungen mit dem Niederschlag, der jeweils einen Monat vor Krankheitsausbruch erfolgte, zeigte ebenso keinen statistischen Zusammenhang.

Diskussion

Historische Aufzeichnungen belegen, dass Milzbrand im Kanton Zürich weit verbreitet war, wobei im Jahr 1894 eine Höchstzahl von Fällen verzeichnet wurde, gefolgt von einem kontinuierlichen Rückgang. Die statistische Analyse zeigte einen signifikanten Zusammenhang zwischen dem Auftreten von Milzbrandfällen und dem Vorhandensein von Gerbereien und wollverarbeitenden Betrieben. Eine Korrelation von rosshaarverarbeitenden Betrieben mit dem Vorkommen von Milzbrand war jedoch – auch im Widerspruch zu anderen Publikationen – nicht feststellbar. Silberschmidt (1896) wies auf den direkten Zusammenhang zwischen einer Rosshaarspinnerei und dem Auftreten von Milzbrand hin, hervorgerufen durch die Düngung einer dem Industriebetrieb benachbarten Wiese mit Abfällen sowie der Belastung der Weiden mit Stäuben aus dem Verarbeitungsprozess.

Ein möglicher Grund für den Rückgang von Milzbrandfällen bei Tieren in den industrialisierten Ländern dürfte das Verbot gewesen sein, infizierte Tierkadaver zu verscharren. Diese zuvor weit verbreitete Praxis stellte ein beträchtliches Risiko für die Verbreitung dar. Das vermehrte Auftreten von Milzbrand in Gemeinden mit wollverarbeitenden Betrieben und Gerbereien, wo Kadaver direkt verscharrt oder auf nahe gelegenen Deponien entsorgt wurden, könnten ein Indiz dafür sein (Schiess, 1997). In der «Vollziehungsverordnung zum Bundesgesetz betreffend die Bekämpfung von Tierseuchen» von 30. August 1920 wird festgehalten, dass die «Einscharrplätze während fünfzehn Jahren weder zum Futterbau noch als Weide benutzt werden dürfen». Da die Sporen von *B. anthracis* äusserst resistent sind, können diese über Jahre oder gar Jahrzehnte überleben und sporadisch Infektionen auslösen. Mehrere Studien legen dar, dass Infektionen oft nach grossen Regenfällen in der warmen Jahreszeit auftreten. Als Grund wurde vermutet, dass *Bacillus*-Sporen nach starken Niederschlägen aus dem Boden geschwemmt werden und sich in Senken ansammeln (Zepezauer, 1980; Lindeque und Turnbull, 1994; Pepper und Gentry, 2002). Im Gegensatz zu anderen Studien konnte aber in unserer Arbeit keine Korrelation zwischen der Zahl der Milzbrandfälle und den meteorologischen Daten, d.h. Niederschlagsmenge oder Durchschnittstemperatur, festgestellt werden.

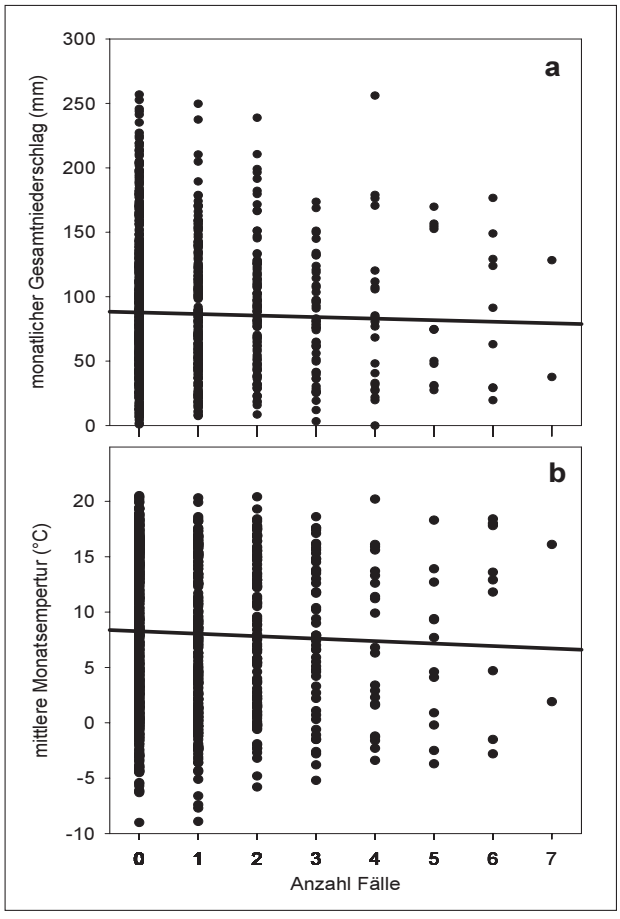

Abbildung 3: Korrelation des Auftretens von Tier-Milzbrand (total 830 Fälle) mit (a) dem monatlichen Gesamtniederschlag und (b) der mittleren Monatstemperatur im Kanton Zürich. a, $r^2=0.000833$; b, $r^2=0.001570$.

Dank

Unser spezieller Dank gilt den Studentinnen und Studenten des Studiengangs «Übersetzen» der Zürcher Hochschule Winterthur, welche im Rahmen einer Projektarbeit den Originaltext aus dem Englischen übersetzt haben. Die Arbeit wurde durch das Amt für Abfall, Wasser, Energie und Luft (AWEL) des Kantons Zürich unterstützt. Wir danken dem Veterinäramt des Kantons Zürich, welches uns Einsicht in das Tierseuchenregister gewährt hat. Ebenso danken wir dem Verband schweizerischer Gerbereien (VSG), dem Textilverband Schweiz (TVS), Bruno Aemissegger (Eskimo Textil AG), Ernst Nef, Romain Rueff und Hanspeter Zingg und der Firma Matousek, Baumann & Niggli AG für die Überlassung von relevanten Informationen.

Cas de charbon dans le canton de Zurich entre 1878 et 2005

On a analysé les mentions historiques de cas de charbon chez des animaux dans le canton de Zurich sur les territoires des communes politiques en examinant l'apparition, le nombre de cas, les espèces d'animaux atteintes et le nombre de communes touchées. Les données ont été corrélées aussi bien avec les activités industrielles (tanneries, traitement de laine et de crins)
dans les communes qu'avec les conditions météorologiques prévalantes. Durant la période examinée, 830 cas de charbon ont été rapportés chez des animaux dans 140 des 171 communes zurichoises ; il s'agissait principalement de bovins. L'apparition de ces cas était corrélée avec les activités industrielles dans les communes concernées. Un rapport positif entre l'apparition de cas de charbon dans une commune et la présence d'entreprise locale qui traitait du matériel potentiellement contaminé comme des peaux, fourrure, laine, poils, viandes ou farines d'os a pu être démontrée. L'influence des exploitations traitant de la laine *(P = 0,004)* et des tanneries *(P = 0,032)* s'est montrée importante alors que les exploitations traitant des crins n'avaient pas d'influence. Il n'y avait pas de rapport statistiquement significatif entre l'apparition de cas de charbon et les conditions météorologiques (précipitations, températures).

Casi di carbonchio tra il 1878 e il 2005 nel canton Zurigo

Le registrazioni storiche sui casi di carbonchio nel Canton Zurigo tra il 1878 e il 2005 in animali sono state analizzate in rapporto ai comuni politici. L'analisi ha preso in conto la comparsa e il numero di casi delle differenti specie di animali malati e il numero dei Comuni colpiti. I dati sono stati messi in relazione con l'attività industriale nei comuni (conciatura, lavorazione della lana e di crine) e con le condizioni meteorologiche prevalenti. Nel periodo preso in esame, in 140 dei 171 comuni zurighesi, sono stati registrati in totale 830 casi di carbonchio in animali (di maggioranza manzi). L'apparizione dei casi è stata correlata con le attività industriali nei rispettivi comuni. E' stata accertata la relazione tra l'apparizione di casi di carbonchio in un comune e la presenza di aziende locali che lavorano materiali potenzialmente contaminati quali pelli, pellicce, lana, peli, carne o farina ossea. Grande si è dimostrato l'influsso delle aziende che lavorano la lana *(P = 0.004)* e delle conciature *(P = 0.032)*, mentre nessun'influenza è stata trovata per quelle che lavorano il crine. Tra l'apparizione di casi di carbonchio e le condizioni meteorologiche (piogge, temperatura) non sono state rilevate relazioni statistiche importanti.

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Chapter II

Detection of bacterial endospores by physico-chemical methods

- Detection of bacterial endospores in soil by terbium fluorescence
- Application of Fourier transform infrared (FTIR) spectroscopy and chemometrical data treatment in microbial ecology: Detection and identification of bacterial endospores

Detection of bacterial endospores in soil by terbium fluorescence

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Keywords
dipicolinic acid; DPA; soil; spore content, terbium fluorescence

Author's contributions
L. Kölle developed the here described fluorescence method for the use with soil samples, and did the first experiments with soil.
A. Brandes Ammann further developed the method, produced the endospore powders from the different *Bacillus* species used for the experiments, and was responsible for the first versions of the manuscript.
H. Brandl, as supervisor, was responsible for the final edition of the manuscript.

Abstract

Aim: The main goal of this study was to investigate the occurrence and distribution of bacterial spores in soils from various locations. In particular, we were interested in the differentiation between different types of soil such as grassland (pasture, meadow), hobby garden, forest, and aquatic sediments.

Methods and Results: Endospores contain high amounts of dipicolinic acid (DPA). In solution, DPA can form a fluorescing chelate complex in the presence of terbium (Tb^{3+}). DPA was released from soil by microwaving or autoclaving and determined by a method based on the fluorescence of the terbium-DPA complex. The highest spore content (up to 10^{10} spores per gram of dry soil) was found in soil from grasslands (e.g. meadow) and in soil depths between 5 and 10 cm.

Conclusions: The method based on terbium fluorescence is fast and easy and can be applied to determine bacterial spores in soil. The addition of aluminium compounds reduces signal quenching by interfering compounds such as phosphate. Spore content is depth-dependent and related to the soil type and the soil carbon-to-nitrogen ratio.

Significance and Impact of the Study: Our study might provide the basis for the detection of "hot spots" of bacterial spores in soil.

Introduction

Spore formation is a survival mechanism of microorganisms when exposed to unfavorable environmental conditions (e.g. nutrient limitation) leading to a "dormant" state (Nicholson 2002; Kennedy *et al.* 1994). A variety of bacteria identified in diverse habitats including soil is able to form endospores. These physiological groups include aerobic heterotrophs (e.g. *Bacillus, Paenibacillus, Brevibacillus, Geobacillus, Thermoactinomyces, Sporolactobacillus*), anaerobes (*Clostridium*, *Anaerobacter, Desulfotomaculum*), microaerophiles (*Sporolactobacillus*), halophiles (*Sporohalobacter*), and phototrophs (*Heliobacterium*, *Heliophilum*) (Sonenshein 2000; Fritze 2004). Bacterial spores are characterized by a series of unique chemical features which can facilitate their identification in natural environments. Besides the high content of minerals (particularly calcium compounds), endospores contain high amounts of dipicolinic acid, DPA (Pendukar and Kulkarni 1988). DPA is uniquely found in bacterial spores in amounts of up to 25% of the spore dry weight and depends on the bacterial species (Murrell 1967; Magge et al. 2008). In solution, a complex is formed in the presence of terbium(III) which shows a very strong and distinctive fluorescence spectrum. Signal strength increases linearly with the DPA concentration (Fig. 1). Originally, DPA was used to detect very low concentrations of terbium (Barela and Sherry 1976). On this basis, methods for the detection of bacterial endospores have been developed (Rosen et al. 1997; Hindle and Hall 1999; Pellegrino et al. 2002; Lester and Ponce 2002; Fichtel et al. 2007, 2008): By the addition of terbium, the DPA content was determined.

However, terbium fluorescence might be interfered by a series of compounds, especially when DPA has to be determined in complex samples such as sediments or soils. It has been reported that the presence of phosphorus compounds (especially *ortho*-phosphate) reduced terbium fluorescence by as much as 98% (Fell et al. 2001). The addition of aluminium compounds (especially aluminium chloride, $AlCl_3$), however, ameliorated the interference caused by the quenching substances (Fell et al. 2001). From a series of organic compounds (benzoate, tryptophan, tyrosine, phenylalanine, glucose, malate, riboflavin, NAD, tryptone) only the latter two (especially tryptone) reduced fluorescence significantly. Carbohydrates (e.g. starch, dextrine) were reported not to interfere with the terbium fluorescence (Chen et al. 2002). Inorganic compounds such as calcium carbonate, sodium chloride, potassium chloride, ammonium sulphate, ammonium nitrate, and sodium nitrate did not lead to a reduction of the fluorescence, but only di-potassium phosphate did (Pellegrino et al. 1998).

Most of endospore-forming bacteria are harmless to humans and animals, but some (e.g. *Bacillus anthracis*) can cause severe diseases such as anthrax. In contrast to many laboratory studies, little is known on the occurrence, frequency, and diversity of bacterial endospores in their

natural soil environment. The aim of this study was to adopt the fluorescence-based method to determine the endospore content in soils from different locations. In particular, we were interested in the differentiation between different types of soil such as grassland (pasture, meadow), hobby garden (flowers, vegetables), forest, and aquatic sediments (border of a stream), the relationship of soil parameters (carbon-to-nitrogen ratio) on the occurrence of bacterial spores, and the distribution of spores in relation to sampling depth.

Materials and methods

Bacterial spores

Different *Bacillus* species (*B. megaterium, B. subtilis*) were cultivated in liquid medium containing (in g/l): glucose (3.6), ammonium chloride (2.5), magnesium sulphate (0.2), calcium chloride (0.07), iron sulfate (0.01), EDTA (0.01), potassium dihydrogen phosphate (0.6), di-potassium hydrogen phosphate (0.9), and yeast extract (1.0). Initial pH was adjusted to 7.0. Erlenmeyer flasks (250 ml) containing 100 ml of growth medium were inoculated and incubated for 10 to 15 days (150 rpm, 30°C). To initiate and stimulate sporulation, bacteria were subsequently transferred to a sporulation medium (identical composition, but without glucose and less ammonium chloride [1 g/l]). After additional 30 days of incubation – until vegetative cells were no longer present after inspection by microscopy – spores were harvested by centrifugation (4000 rpm, 4°C, 10 min), washed twice with ultrapure water and immediately frozen in liquid nitrogen followed by lyophilization.

Soil samples

Samples from different locations were collected using a stainless steel soil corer (15 mm in diameter), which was sterilized by flaming in ethanol before each sampling. Cores with a maximum length of 25 cm were obtained, cut in sections of 5 cm, transferred to sterile screw cap Falcon tubes (20 ml), and stored on dry ice. After return to the laboratory, samples were immediately lyophylized or stored at -80°C until further processing.

Sampling sites were located in the surroundings of Zurich (Switzerland): grassland soil, meadow (Männedorf; Uerikon; Stäfa; Dübendorf), hobby garden (Zurich, Irchel campus, University of Zurich), pasture (Zurich, Irchel campus, University of Zurich; Wädenswil), forest soil (Stäfa), aquatic sediments (river Glatt in Dübendorf). A map is shown in Fig. 2. Average annual rainfall in this region is about 1100 mm (MeteoSchweiz).

Lyophilized aliquots of approximately 1 g were transferred to an Eppendorf micro test tube (2 ml) and milled (by adding a 6mm glass bead) in TissueLyser (Retsch, Haan, Germany) for 5 x 1

min. Elemental composition (carbon, hydrogen, nitrogen) of soil was performed with a CHN-932 elemental analyzer (Leco Corp., St. Joseph, MI, USA). Approximately 10 mg of powdered soil was used for analysis. Phosphate in aqueous soil extracts (250 mg soil in 5 ml sodium acetate buffer; 0.2 M, pH 5) was determined using commercially available kits (LCK 348 and 349; Hach Lange AG, Hegnau, Switzerland).

Release of DPA from endospores

10 mg of dry endospore powder was resuspended in 10 ml sodium acetate buffer (0.2 M, pH 5). Spores were counted under the microscope using a Neubauer counting chamber. Soil samples were thawed and 50 mg were suspended in 0.9 ml sodium acetate buffer and 0.1 ml aluminium chloride ($AlCl_3$, 0.5 M). Samples were microwaved (Berghof Microwave Digester MWS-1, with built-in *in situ* infrared temperature control) in Teflon TFM screw cap digestion vessels. Temperature and power was set to 140°C and approximately 680 W (80%), respectively.

Alternatively, DPA was released from spores by autoclaving the samples in screw cap glass test tubes for 15 minutes at 121°C. The presence of endospores after microwaving and autoclaving was determined by microscopy. The identical protocol was applied for soil samples.

Fluorescence measurement

After cooling for 30 minutes, 100 µl of the endospore suspensions were mixed with 100 µl terbium chloride solution ($TbCl_3$, 30 µM) in white 96-well microtiter plates (in 8 replicates). Fluorescence was immediately measured using a plate reader (SpectraMax M2, Bucher Biotec, Basel, Switzerland) with the following settings: time resolved fluorescence (delay 50 µs, interval 1200 µs) at an excitation wavelength of 272 nm, emission wavelength of 545 nm, 10 endpoint readings per sample at 30°C. The number of spores in the soil samples was determined using standard addition method with spores of *B. subtilis* (Fichtel et al. 2007). Spore content was expressed as equivalents of *B. subtilis*.

Results

Elemental composition of soil (in % of dry soil) varied between 2.2 and 15.4, 0.2 to 1.4, and 0.2 to 2.0 for total carbon, total hydrogen, and total nitrogen, respectively.

Microwave treatment of endospore suspensions led to a fast release of DPA (Fig. 3). Within two minutes, maximum release was obtained. Increased treatment times did not improve DPA mobilization. Bacterial spore content was dependent on soil type (Fig. 4). Highest spore numbers up

to (4 x 10^8 spores per gram dry soil) were found in agriculturally used land (grassland soil: hobby garden, meadow, pasture), less in forest soil. Aquatic sediments showed lowest spore numbers.

A transect (approximately 100 m in length) through a field with different land use management (unused meadow, hobby garden, pasture) gave spore numbers in the range of 5 to 9 x 10^8 endospores per gram of dry soil (Fig. 5). Spore counts were independent of the type of land use: In hobby garden soil, counts were not significantly different from soil samples taken from a pasture (P=0.423; t-test).

Depth distribution of endospores from an area currently used as hobby garden showed the highest numbers in a horizon of 5 to 10 cm (Fig. 6). The two methods evaluated (microwaving, autoclaving) for the mobilization of DPA from bacterial spores gave similar results (Fig. 6). However, microwaving was less time-consuming, whereas autoclaving allowed faster throughput of samples.

Spore content was correlated to the carbon-to-nitrogen ratio (Fig. 7). At C/N ratios >20 only low spore counts (0.5 x 10^8 spores per gram of dry soil) were detected as compared to C/N ratios <20.

Discussion

The method based on terbium fluorescence for the detection of bacterial endospores in soils is fast and easy. DPA is released from spores within two minutes when samples are processed by microwaving. There is no further increase of the fluorescence signal with prolonged microwaving times (Fig. 3). The method determines total endospore numbers, but is not species-specific.

The interference of different compounds present in soil (e.g. phosphate) might lead to quenching of the fluorescence signal. This drawback can be overcome by the addition of aluminium chloride as already shown for the determination of bacterial spores in aquatic sediments (Fichtel et al. 2008). Concentration of *ortho*-phosphate in soil extracts (22.5 µM) was reduced by the addition of aluminium chloride to concentrations below the detection limit (<1.2 µM). Concomitantly, a de-colorization of the extract was observed suggesting the removal of humic acids which have also the potential to form complexes with terbium and quench the fluorescence signal (Fichtel et al. 2007).

Regarding the different sampling sites, our results show that grassland soils (meadow, hobby garden, pasture) contains much more bacterial spores than forest soil and aquatic sediment. Endospore content was dependent on the C/N ratio. It has been demonstrated in pure cultures of *Bacillus thuringiensis* in a stirred bioreactor that low carbon-to-nitrogen ratios of 4:1 resulted in high spore counts (Farrera et al. 1998). In contrast however, endospore formation in *Streptomyces coelicolor* was stimulated under nitrogen-limiting conditions (Karandikar et al. 1996). In particular,

C/N ratios between 50 and 100 promoted sporulation, whereas C/N rations <40 did not allow spore formation. Our results showed, that in soils with extremely high C/N ratios, endospore content was low.

Regarding the sporulation of fungi – although they do not contain DPA – the importance of C/N ratio was stressed by Gao and co-workers (Gao et al. 2007). A carbon-to-nitrogen (C/N) ratio of 20 stimulated spore formation by fungi such as *Penicillium camembertii* (Krasniewski et al. 2007). The fungus *Colletotrichum coccoides* produced highest spore counts at a C/N ratio of 5 to 10, whereas at a ratio of 40 spore formation was significantly lower (Yu et al. 1998). Similarly, in *Plectosporium tabacinum* optimal spore formation was found when C/N rations were between 5 to 10 (Zhang et al. 2001).

The distribution of endospores in marine sediments (determined as DPA) showed only a low correlation with the content of total organic carbon and varied with the sediment type (Fichtel et al. 2008). Highest numbers were found in organic-rich black sediments, lowest number in sandy sediments.

It was hypothesized from anthrax outbreaks, that the high numbers of *Bacillus* might be related to soils rich in organic matter, i.e. to a high C/N ratio (Dragon et al. 2001). These soil environmental conditions are suggested to support the presence and viability of *B. anthracis* spores (Dragon and Rennie 1995). We could not confirm this hypothesis.

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Figure legends

Figure 1. Fluorescence signal strength correlated to dipicolinic acid (DPA) concentration.

Figure 2. Sampling sites in the surrounding of Zurich (Switzerland). Solid squares denote single sites.

Figure 3. Release of dipicolinic acid (DPA) from a bacterial spore suspension of *B. megaterium* as a function of microwave treatment. Points represent mean values of 8 replicates.

Figure 4. Endospore content (expressed as equivalents of *B. subtilis*) of soil from ten different locations. A: grassland soil (from Männedorf, location 1); B: grassland soil (from Männedorf, location 2); C: grassland soil (from Männedorf, location 3); D: grassland soil (from Uerikon); E: grassland soil (from Dübendorf); F: forest soil (from Stäfa, location 1); G: forest soil (from Stäfa, location 2); H: forest soil (from Stäfa, location 3); I: aquatic sediment (river Glatt in Dübendorf, location 1); J: aquatic sediment (river Glatt in Dübendorf, location 2). Bars represent mean values of triplicates. See also Fig. 2 for location.

Figure 5. Transect of 100 m through a field showing different land use management: unused meadow (m), hobby garden, pasture. Bacterial spore content is expressed as equivalents of *B. subtilis*. Data represent mean values of triplicates.

Figure 6. Depth profiles (0 to 25 cm) of bacterial spore content in soil from a pasture. Comparison of autoclaving (open bars) and microwaving (solid bars) to release dipicolinic acid (DPA). Bars represent mean values of triplicate samples. * sample was lost during filtration step.

Figure 7. Endospore number (expressed as equivalents of *B. subtilis*) as function of soil carbon-to-nitrogen ratio.

Fig. 1

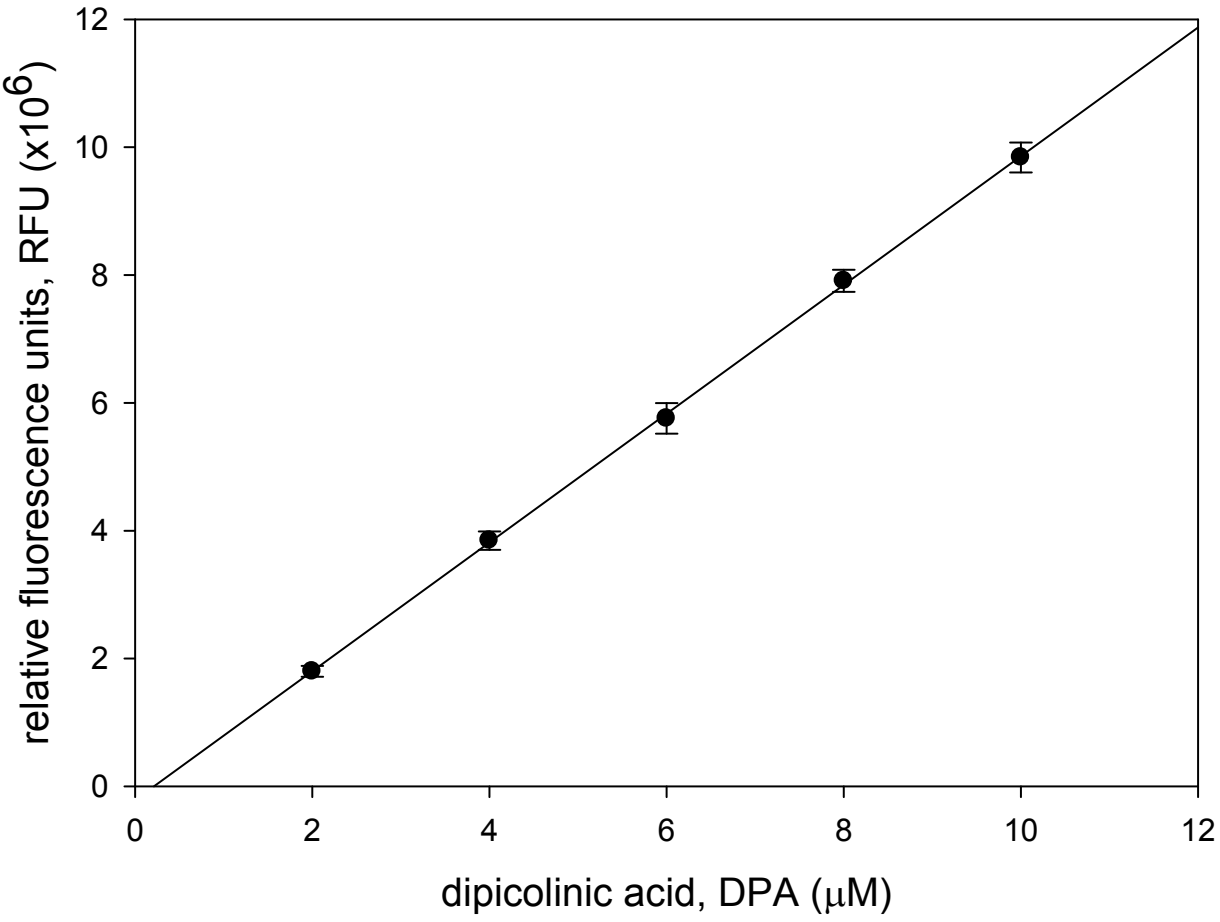

relative fluorescence units, RFU (x10^6)
dipicolinic acid, DPA (μM)
0
2
4
6
8
10
12

Fig. 2

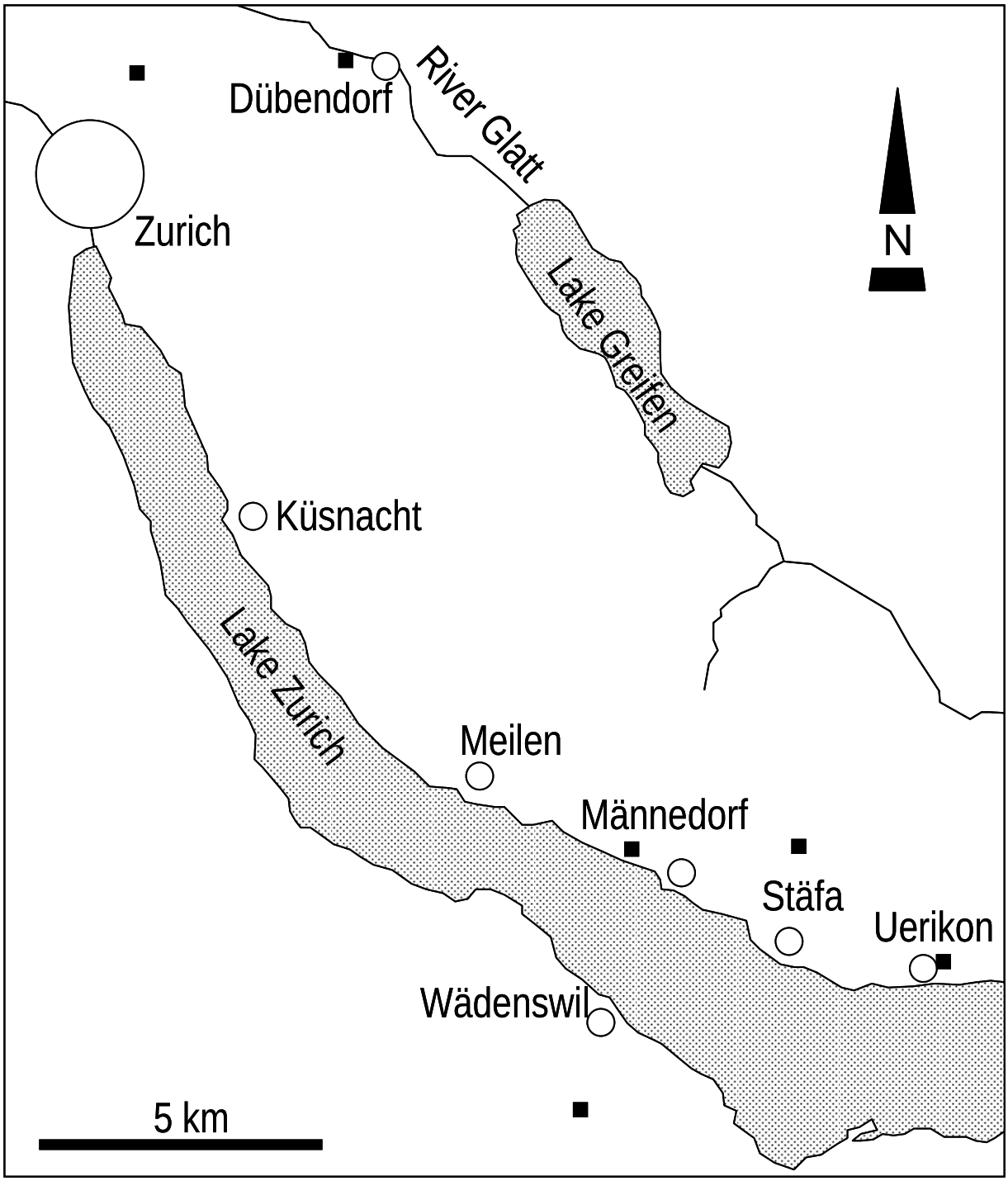

Dübendorf
River Glatt
Zurich
N
Lake Greifen
Küsnacht
Lake Zurich
Meilen
Männedorf
Stäfa
Uerikon
Wädenswil
5 km

Fig. 3

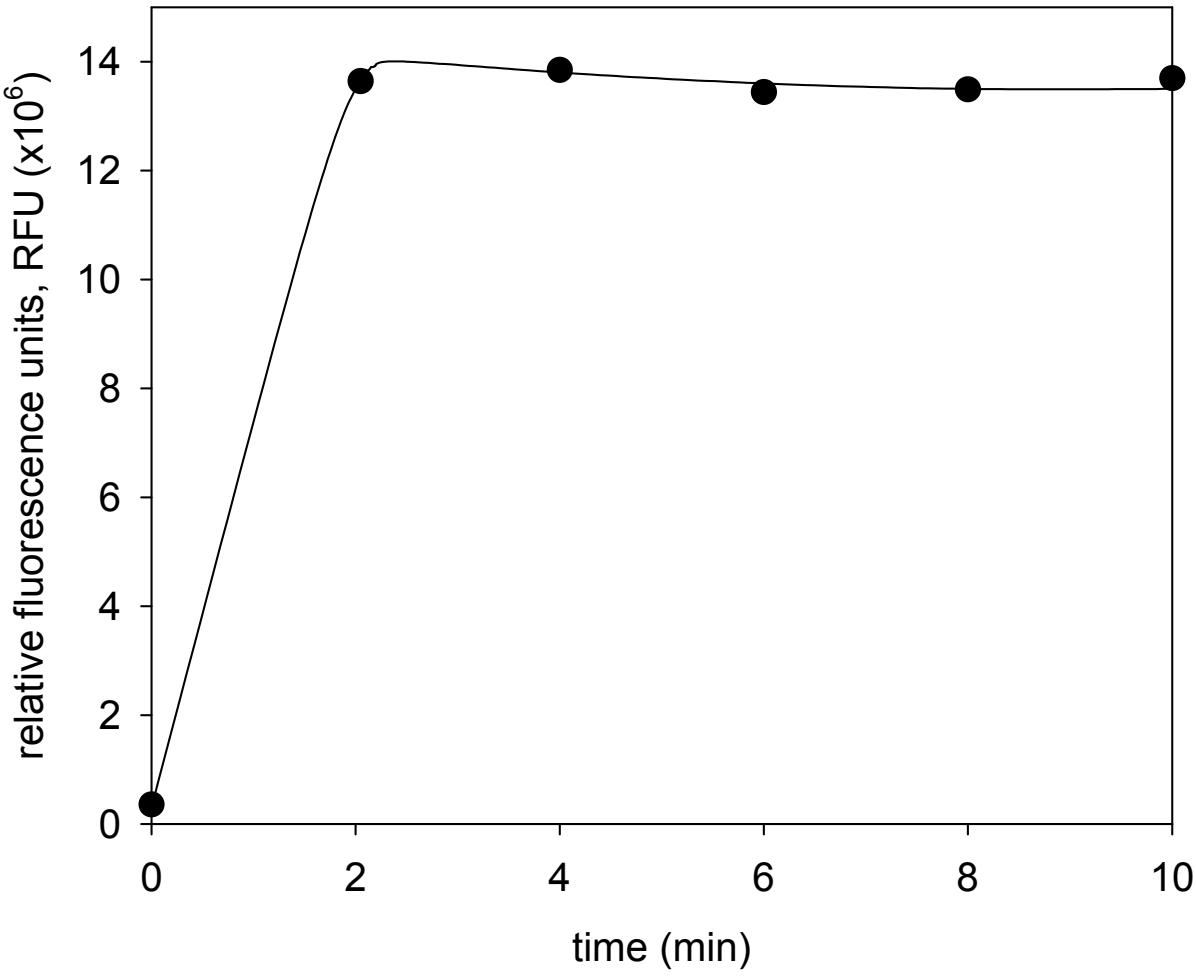

relative fluorescence units, RFU (x10^6)
14
12
10
8
6
4
2
0
0
2
4
6
8
10
time (min)

Fig. 4

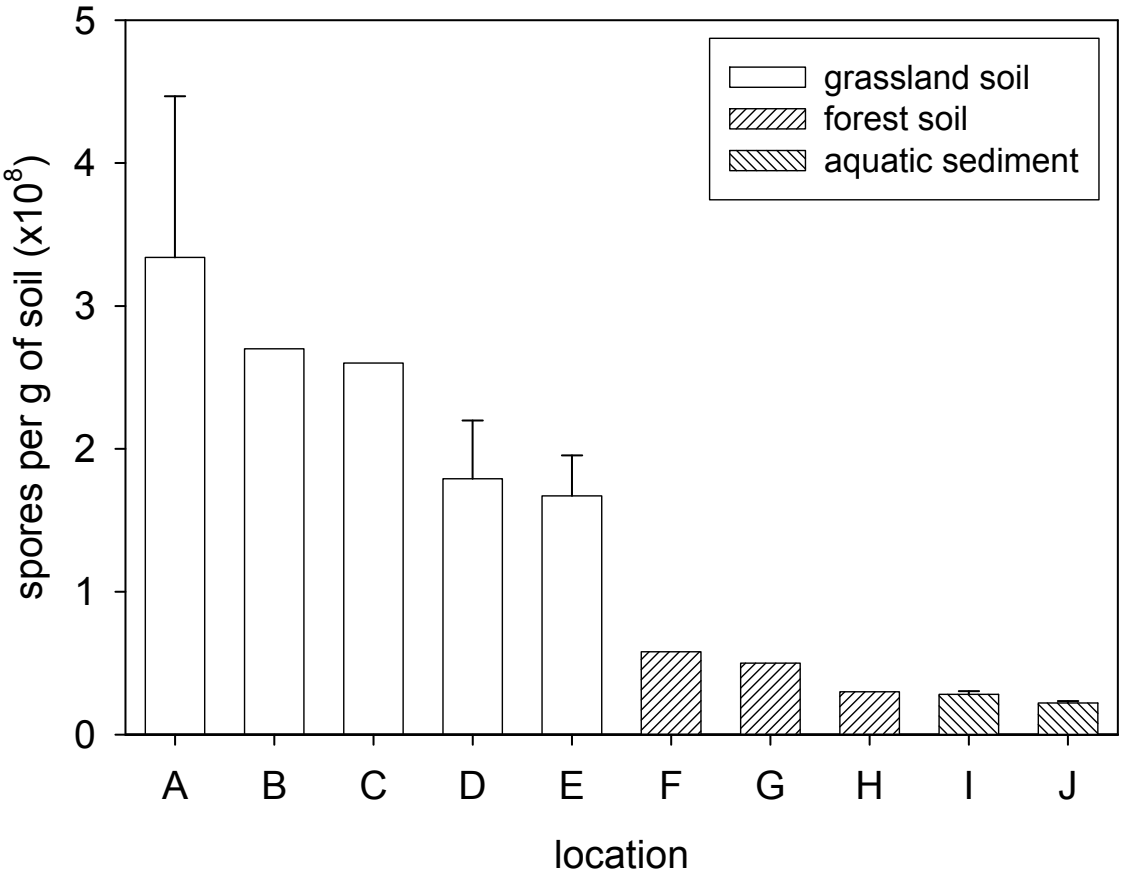

5
4
3
2
1
0
spores per g of soil (x10^8)
grassland soil
forest soil
aquatic sediment
A
B
C
D
E
F
G
H
I
J
location

Fig. 5

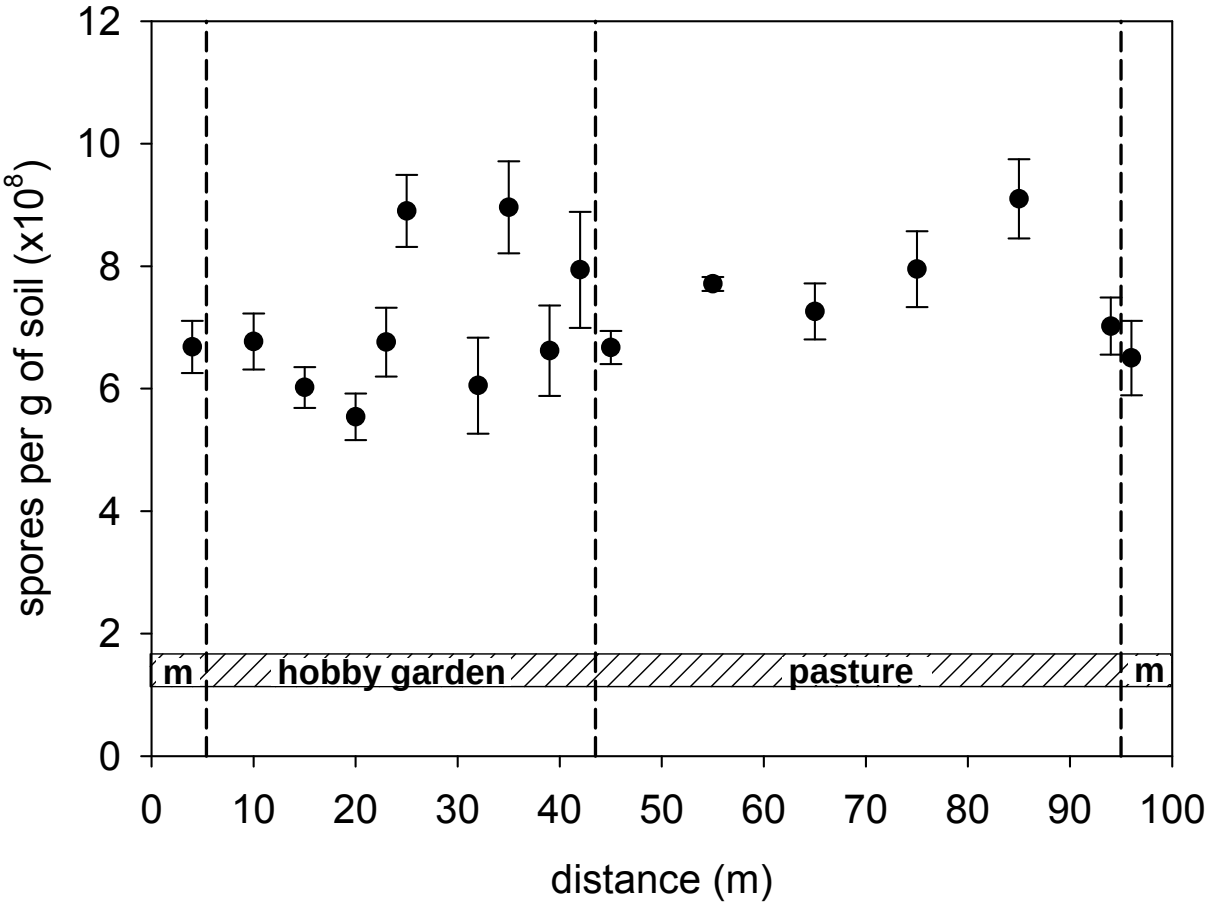

spores per g of soil (x10^8)
12
10
8
6
4
2
0
m
hobby garden
pasture
m
0
10
20
30
40
50
60
70
80
90
100
distance (m)

Fig. 6

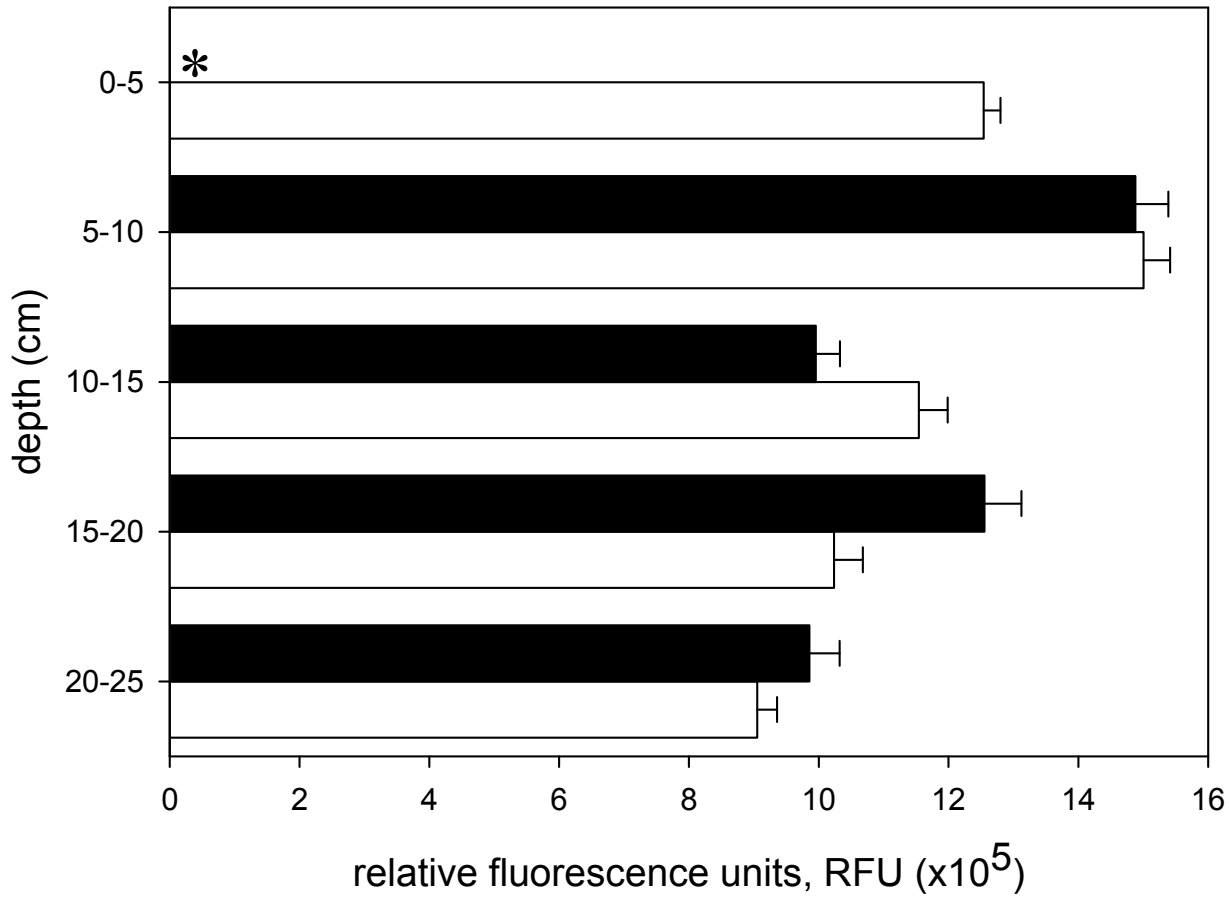
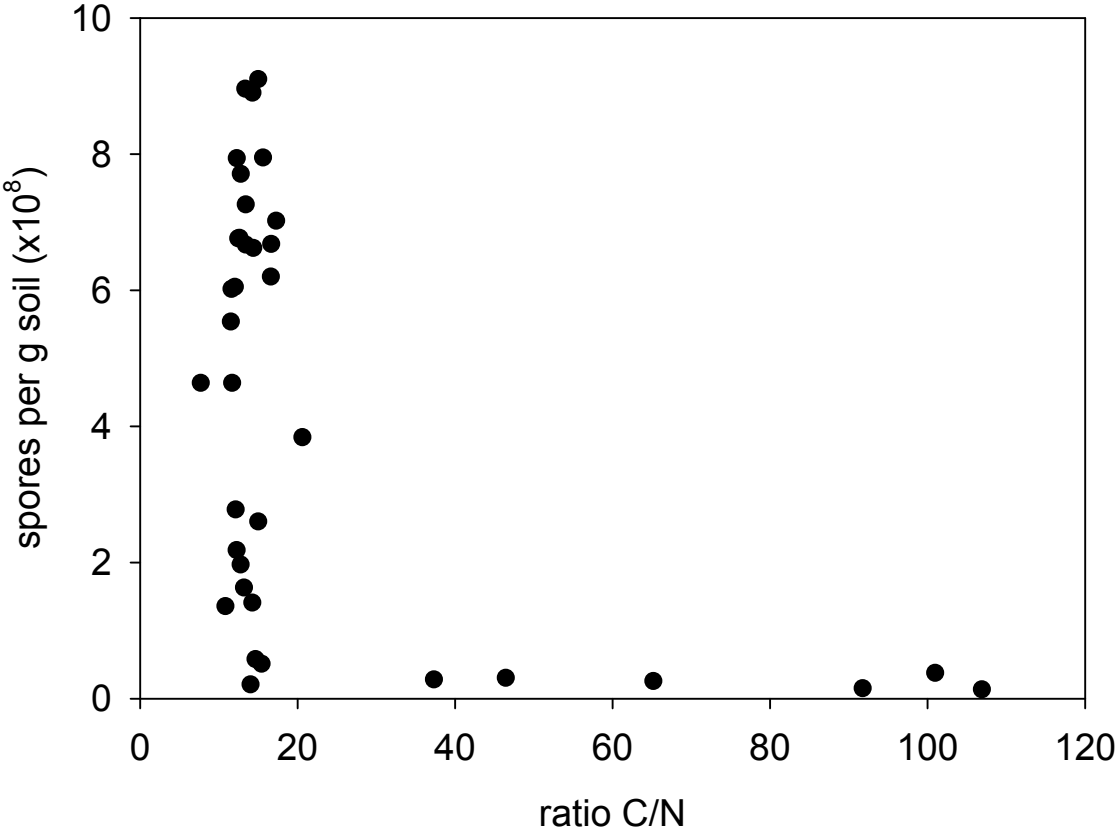

*
0-5
5-10
10-15
15-20
20-25
depth (cm)
0
2
4
6
8
10
12
14
16
relative fluorescence units, RFU (x10^5)

Fig. 7



Application of Fourier transform infrared (FTIR) spectroscopy and chemometrical data treatment in microbial ecology: Detection and identification of bacterial endospores

Abstract

Fourier transform infrared (FTIR) spectroscopy has been used routinely as analytical tool in chemistry for many years. Only a few years ago its application started in food industry for hygiene measurements. In microbial ecology little work has been done so far. FTIR can be applied as a rapid and non-invasive method to detect and identify microorganisms. The aim of this study was to develop a fast and reproducible non-molecular method to identify bacteria in environmental samples. The specific and fingerprint-like spectra allow discrimination - under optimal conditions - down to the species level. At present, this is possible for pure or mixed cultures without matrices such as soil. Spores from pure cultures of seven different *Bacillus* strains (*B. atrophaeus, B. brevis, B. circulans, B. lentus, B. megaterium, B. subtilis, B. thuringiensis*) in the presence of different solid matrix substances such as clay minerals (bentonite), milk powder, powdered mushrooms, or dry herbs as well as mixtures of matrix compounds and bacterial strains at various wt/wt ratios have been investigated with reflection- and transmission methods. Hierarchical cluster analysis and multidimensional scaling of the second derivation of the spectra let discriminate different species and spores-matrix-mixtures. At present the method is applicable for pure cultures and simple matrices. For complex samples as soil discrimination is not possible.

Introduction

Fourier transform infrared (FTIR) spectroscopy is a rapid and non-invasive physico-chemical method to detect and identify microorganisms or specific cell components. Whole living cells can be analysed without destruction, which allows in vivo investigations. The basic principle is relatively simple. An infrared beam excites the molecules and atoms in a sample to vibrate and rotate. Through a mathematical transformation the sum of all these movements can be made visible as a absorption spectrum which is dependent on the chemical components and for this reason specific for the sample. The spectra of biological samples can be divided in different regions or windows. Between wavenumber (wn, cm^{-1}) 650 and 1800 is the typical fingerprint region for organisms (typical FTIR absorption spectra of *Bacillus* endospores are shown in the appendix of this thesis). Basic principles and application possibilities in biology are described in Naumann (1990). Burgula *et al.* (2007) report W. W. Coblentz to be the very first who analysed biological samples with infrared spectroscopy already in 1911. First biological experiments with infrared spectroscopy have been conducted in the early fifties. Most of them were in a general sense (Stevenson &

Bolduan 1952; Levine *et al.* 1953; Thomas & Greenstreet 1954; Riddle *et al.* 1956; Kull & Grimm 1956a; Greenstreet & Norris 1957; Rideal & Adams 1957; Haynes *et al.* 1958; Kenner *et al.* 1958; Norris & Greenstreet 1958) and only a few for application in medicine/diagnostics or food production (Levine *et al.* 1955; Kull & Grimm 1956b; Bartlet 1957). At that time the instruments and possibilities for further data processing have been simpler than today. In the eighties Dieter Naumann and co-workers (Naumann *et al.* 1991) developed in a joint project of the Robert Koch Institute in collaboration with a manufacturer of FTIR spectrometers (Bruker, Germany) efficient methods and instruments for microbiological use. Until today, methods – especially data processing – have been further developed to get faster and easier better results. Most research is done with pure cultures, for example in general or environmental studies, related to medicine and diagnostics or food, and with biofilms or relatively simple matrices (see Table 1). Mariey and colleagues (2001) give a short summary of publications of the nineties concerning the discrimination, classification and identification of microorganisms as whole cells. Our aim was to detect and identify bacteria, especially bacterial spores, in more complex environmental samples like soil.

Material and Methods

Bacterial spores: Fife *Bacillus* type strains were purchased from German Collection of Microorganisms and Cell Cultures (*Bacillus atrophaeus* DSMZ 7264, *Brevibacillus brevis* DSMZ 30, *Bacillus circulans* DSMZ 11, *Bacillus lentus* DSMZ 9, *Bacillus thuringiensis* DSMZ 2046). *Bacillus subtilis* was obtained from Andermatt Biocontrol AG, Stahlermatten 6, CH-6146 Grossdietwil, www.biocontrol.ch (Biopro) and Bio-Protect Gesellschaft für biologischen Pflanzenschutz mbH, Lohnerhofstrasse 7, D-78467 Konstanz www.bio-protect.de (clone BD 170). *Bacillus megaterium* was from our institute. Bacilli from DSMZ were cultured in liquid medium 1 as suggested by DSMZ. *B. megaterium* and *B. subtilis* were cultured in liquid medium (1.8 g/l base medium, 1 ml/l trace elements [see Table 2], 1 g/l yeast extract, 50 mM glucose, 50 mM ammonium chloride, pH 7). They all were incubated in a shaker (Kühner AG Lab-Therm incubator shaker) at 150 rpm and 30°C for 10 – 15 days. To initiate and force the sporulation, bacteria were then transferred to the sporulation-medium (without glucose and less ammonium chloride [20 mM]). After a further 30 days, approximately, the spores were harvested by centrifugation, washed to remove media compounds and subsequently freeze dried.

Samples: The following samples were measured: pure spore powder, dried vegetative cells of *B. circulans*, grinded bentonite (clay minerals), milk powder, grinded dried kitchen herbs, grinded dried white mushroom and mixtures of bacterial spores and bentonite at various wt/wt ratios (1:1, 1:2, 1:4, 1:6, 1:8, 1:10).

FTIR: A JASCO 4200-FTIR was used. Measurements with reflection method were performed in the attenuated total reflection (ATR) mode using an ATR accessory equipped with a diamond or a

zinc selenide (ZnSe) prism. A small amount of sample, enough to cover the crystal/prism, was placed onto the ATR and spectra were collected. Settings for diamond prism: Scans: 100, Resolution: 2 cm^{-1}, Zero Filling: On, Apodization: Cosine, Gain: Auto (32), Aperture: Auto (5 mm), Scanning Speed: Auto (2 mm/sec), Filter: Auto (30000 Hz) , Background: Air. Settings for ZnSe prism: Scans: 50, Resolution: 4 cm^{-1}, Zero Filling: On, Apodization: Cosine, Gain: Auto (8), Aperture: Auto (7.1 mm), Scanning Speed: Auto (2 mm/sec), Filter: Auto (30000 Hz) , Background: Air. For transmission the samples were suspended in ultra pure water (18.2 mΩ) and 100 µl air dried on a polyethylene (PE) film in a slide rim. Spectra were collected with the same settings as with the ZnSe prism (Background: PE film). Each sample was measured three times. Measurement range was 4000 – 650 cm^{-1} for ATR and 4000 – 400 cm^{-1} for transmission.
Data processing and analysis: Raw spectral data were processed with JASCO Spectra Manager Version 2. First all spectra were normalized as follows: 1. ATR-correction (for reflection only), 2. smoothing (Savitzky-Golay, width = 7 for diamond, width = 15 for ZnSe and transmission), 3. truncate (3000 – 650 cm^{-1} for diamond, 1800 – 650 cm^{-1} for ZnSe, none for transmission), 4. baseline correction (linear, 0/0). Afterwards the second derivative was calculated (Savitzky-Golay, width = 7 for diamond, width = 5 for ZnSe, width = 15 for transmission). As next step hierarchical cluster analysis (*hclust*, Ward's minimum variance method) and principal coordinates analysis (*cmdscale*) or principal components analysis (*prcomp*) were performed with the open source software package R (R Development Core Team 2008). For principal coordinates analysis the mean of the three datasets per sample was taken.

Results

FTIR in both the reflection and the transmission mode produced useful results.
Reflection: With the diamond only pure spore powders and the dried vegetative cells of *B. circulans* were measured. As shown in Fig. 1 and Fig. 2 discrimination of the different *Bacillus* species, spores and vegetative cells was possible. With the ZnSe crystal mixtures of spores and bentonite were measured in addition to pure samples. Here as well clusters were formed (Fig. 3).
Transmission: In the transmission mode pure spore powders of all five species were measured and discriminated as shown in Fig. 4 and Fig. 5. Discrimination of spores of three different *Bacillus* species (*B. atrophaeus, B. megaterium, B. subtilis*) in mixtures with bentonite at various ratios was possible as well (Fig. 6, Fig. 7, Fig. 8).

Discussion / Conclusions

FTIR is a rapid and easy method to detect microorganisms. No complex and often time-consuming preparation of the samples is required. For ATR measurement (reflection) solid or liquid samples can directly be placed on the crystal. Transmission requires suspension for solids and always a

drying step before measurement. Another advantage of FTIR is the small amount of sample to be used and as the sample does not get dissipated by the method there is theoretically nearly no loss. Furthermore FTIR is a relatively inexpensive method, apart from the spectrometer no other material or consumables have to be purchased.

Most of the studies done so far used the transmission method. Only at the end of the nineties the application of ATR begun but still to a minor degree. Nevertheless Baldauf and co-workers (Baldauf *et al.* 2006) found ATR to give the best resolution of peaks by using the least amount of biomass compared to other FTIR methods.

As our results and several studies show, discrimination of biological samples with FTIR and subsequent chemometrical analysis of the spectra is possible. An unknown sample can be identified by assigning it to a cluster of known samples. The more known samples are available, the more reliable is the identification of unknowns. Foster *et al.* (2004) performed similar experiments with different *Bacillus* species (vegetative cells and spores) and bentonite and tryptic soy broth as nonbacterial samples. They used the transmission method with a four-step statistical model and had comparable results. Subramanian and colleagues (Subramanian *et al.* 2006) presented a clear separation of vegetative cells, live spores and autoclaved spores using the ATR method and SIMCA (soft independent modelling by class analogy). Very recently Forrester *et al.* (2009) published their results on discrimination and identification of endospores of five different *Bacillus* species, all grown and sporulated under the same standardized conditions, using FTIR spectra, principal components analysis and Mahalanobis distance. Organisms in mixtures cannot be identified one by one with the method applied in our study. But Yu & Irudayaraj (2006) developed a mathematical procedure which allowed the identification of a three-organism-mixture out of five possible bacteria (liquid cultures, 10^9 CFU/ml each). If the matrix is complex, for example soil, then the background is too high or the spectrum too complex to find the essential peaks for a certain organism. This problem could probably be solved with the above mentioned mathematical procedure or a further development of it. But still the microorganism(s) looked for might have to be present in a relatively high concentration.

The trees resulting from hierarchical cluster analysis of pure cultures are comparable to phylogenetic trees derived from classical methods (Fig. 2, Fig. 5). This supports the findings of other studies (Helm *et al.* 1991a; Haag *et al.* 1996; Kirschner *et al.* 2001).

In principle, data processing could be automated, which would make the method even simpler. There are fully automated devices available to identify chemical substances, especially hazardous materials. These instruments work with reference libraries and compare primary spectra or parts of them without further processing. We also conducted preliminary investigations to establish a biological reference library. But this, too, only works with nearly pure cultures or organisms in a simple matrix and in relatively high concentrations. As another problem cultivation conditions influence the spectrum of an organism (Norris & Greenstreet 1958; Curk *et al.* 1994; Haag *et al.*

1996; Rodriguez-Saona *et al.* 2001; Filip *et al.* 2004; Baldauf *et al.* 2007). For spore powders these influences are less than for vegetative cells. When studying vegetative cells the age of the culture has to be considered, too. Although in Lai *et al.* (2004) culture age did not affect the recovery of *Carnobacterium* strains. There already exist commercial data bases of microorganisms, but they are mostly related to instruments of specific manufacturers (e.g. Bruker Optics). And they are applicable for pure cultures only, cultivated under standardized conditions. Some research has been and is still done on the use of artificial neural networks (ANNs) and spectral data bases for the identification of organisms (Goodacre *et al.* 1996; Goodacre *et al.* 1998; Goodacre *et al.* 1998; Schmitt *et al.* 1998; Kirschner *et al.* 1999; Udelhoven *et al.* 2000; Rebuffo *et al.* 2006; Rebuffo-Scheer *et al.* 2007b; Bosch *et al.* 2008). Detection and classification of microorganisms with FTIR in combination with a reference library could be a promising approach for medical diagnostics. This is also concluded by Maquelin *et al.* (2002) who made a review on the application of vibrational spectroscopy in this field. In food industries, where much research for the application of FTIR is done and some methods already applied, especially in dairy and meat products, the range of organisms of interest is not that wide. Mostly samples are screened to determine the presence of one or a few specific harmful organisms. This simplifies the application of the method. In ecology a successful application of FTIR depends on the field of interest. Similar to Kos *et al.* (2002), who developed a screening method for the determination of *Fusarium* fungi on maize, a genus including agriculturally important toxigenic species causing great damage in the food and livestock industry, our group also used FTIR to distinguish grasses infected with an endophytic fungi from non-infected (Brandl *et al.* 2009). In general it can be stated that investigations on organism level in soil ecology may be difficult without combination with other methods to prepare the samples due to strong matrix effects. For the detection and identification of microorganisms the method shows a much better applicability with water or air samples. But for studying microbial community structures there might also be a potential for FTIR with soil. As an example microbial succession in earthworm casts have been investigated by Scullion and co-workers (Scullion *et al.* 2003a; Scullion *et al.* 2003b). The future of FTIR and microorganisms or other cells probably lies in FTIR microspectroscopy, where selective spectra of single cells can be taken. Some promising research results have already been published (Ngo Thi *et al.* 2003; Mossoba *et al.* 2005; Perkins *et al.* 2005; Cheung *et al.* 2007; Rebuffo-Scheer *et al.* 2007a).

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Tables & Figures

Table 1: Examples for applications of FTIR spectroscopy in biological research

Reference	Organisms	Field
Al-Holy *et al.* 2006	*Bacillus cereus, Salmonella enterica, E. coli, Listeria*	food
Al-Qadiri *et al.* 2008 (NIR)	bacterial milk spoilage	biofilms, simple matrices, food
Amiel *et al.* 2000	*Lactobacillus, Lactococcus, Leuconostoc, Streptococcus*	food
Baldauf *et al.* 2006	*Salmonella enterica*	food
Baldauf *et al.* 2007	*Salmonella enterica*	food
Beattie *et al.* 1998	*Bacillus*	food
Bosch, *et al.* 2008	nonfermenting gram-negative rods	medicine/diagnostics
Bosch *et al.* 2006	*Bordatella pertussis*	biofilms, simple matrices
Cheung *et al.* 2007	*Pseudomonas aeruginosa*	biofilms, simple matrices
Cheung *et al.* 1999	*Bacillus subtilis*	general, environment
Crupi *et al.* 2004	*human tissue*	medicine/diagnostics
Curk *et al.* 1994	*Lactobacillus*	food
Filip & Hermann 2001	*Pseudomonas*, other soil bactria	general, environment
Filip *et al.* 2004	*Bacillus subtilis*	general, environment
Foster *et al.* 2004	*Bacillus* spores	general, environment
Garip *et al.* 2009	*Bacillus, Micrococcus*	food
Goodacre *et al.* 1998	*Staphylococcus aureus*	medicine/diagnostics
Goodacre *et al.* 2000	*Bacillus* spores	general, environment
Goodacre *et al.* 1996	*Streptococcus, Enterococcus*	medicine/diagnostics
Goodacre *et al.* 1998	bacterial isolates associated with urinary tract infection (*E. coli, Proteus mirabilis, Klebsiella, Pseudomonas aeruginosa, Enterococcus*)	medicine/diagnostics
Guibet *et al.* 2003	*Enterococcus*	medicine/diagnostics
Haag *et al.* 1996	*actinomycetes*	general, environment
Helm & Naumann 1995	*Bacillus, Clostridium*	general, environment
Helm *et al.* 1991a	*Entrobacteriaceaea, Aeromonas, Pseudomonas, Staphylococcus, Streptococcus, Clostridium, Legionellai*	general, environment
Helm *et al.* 1991b	*Entrobacteriaceaea, Aeromonas, Pseudomonas, Staphylococcus, Streptococcus, Clostridium, Legionellai*	general, environment

Table 1 continued

Reference	Organisms	Field
Kirschner *et al.* 2001	*Enterococcus*	general, environment
Kirschner *et al.* 1999	*Enterococcus Staphylococcus*	medicine/diagnostics
Kos *et al.* 2002	*Fusarium graminearum*	biofilms, simple matrices
Kümmerle *et al.* 1998	food-born yeasts	food
Lai *et al.* 2004	*Carnobacterium, Lactobacillus, Enterococcus durans, Lactobacillus maltaromicus, Vagacoccus salmoninarum*	food
Lin *et al.* 1998	*Bacillus*	food
Mannig *et al.* 2008	*Salmonella enterica*	food
Melin *et al.* 2004	*Mycoplasma, Ureaplasma, Acholeplasma*	medicine/diagnostics
Mossoba *et al.* 2003	*E. coli, Pseudomonas aeruginosa, Bacillus cereus, Listeria innocua*	food
Mossoba *et al.* 2005	*Yersinia, Staphylococcus, Salmonella, Listeria, Enterobacter, Citrobacter, Klebsiella, Escherichia*	food
Mossoba *et al.* 2002	*E. coli, Pseudomonas aeruginosa, Bacillus cereus, Listeria innocua*	general, environment
Naumann *et al.* 1988	clinically relevant gram-positive and gram-negative bacteria	medicine/diagnostics
Ngo Thi & Naumann 2007	*Legionella bozemanii, Bacillus megaterium, Candida albicans*	biofilms, simple matrices
Ngo Thi *et al.* 2003	*Baccillus, , Citrobacter, E. coli, Enterobacter, Staphylococcus, Stretococcus, Pseudomonas, Candida*	general, environment
Nichols *et al.* 1985	*E. coli, Bacillus, Pseudomonas, Staphylococcus, Clostridium, Vibrio, Methylobacterium, Methyosinus, Nitrobacter*	biofilms, simple matrices
Oberreuter *et al.* 2003	bacterial cheese surface flora	food
Oberreuter *et al.* 2000	*Saccharomyces cerevisiae, Hanseniaspora uvarum, Lactobacillus acidophilus, Streptococcus salivarius*	food
Oberreuter *et al.* 2002	*Micrococcineae and Corynebacterineae*	general, environment
Oust *et al.* 2004	*Lactobacillus sakei, L. plantarum, L. curvatus , L. paracasei*	food
Perkins *et al.* 2005 (micro)	*Bacillus* and *Clostridium* spores	general, environment
Preisner *et al.* 2007	*Enterococcus faecium* (VRE)	medicine/diagnostics
Rebuffo *et al.* 2006	*Listeria*	food
Rebuffo-Scheer *et al.* 2007a	*non-tubercoulous mycobacteria*	medicine/diagnostics
Rebuffo-Scheer *et al.* 2007b	*Listeria*	food

Reference	Organisms	Field
Riedel *et al.* 1996	microorganisms from clean rooms	general, environment
Rodriguez-Saona *et al.* 2001 (FT-NIR)	*E. coli, Bacillus amyloliquifaciens, Pseudomonas aeruginosa, Bacillus cereus, Listeria innocua*	biofilms, simple matrices
Rodriguez-Saona *et al.* 2002 (FT-NIR)	*E. coli, Bacillus, Pseudomonas aeruginosa, Listeria innocua*	biofilms, simple matrices
Rodriguez-Saona *et al.* 2004 (FT-NIR)	*E. coli, Bacillus, Pseudomonas aeruginosa*	biofilms, simple matrices
Schmitt & Flemming 1998	bacterial biofilms in drinking water systems	biofilms, simple matrices
Schmitt *et al.* 1998	*Pseudomonas aeruginosa,* bacterial mix from potable water	biofilms, simple matrices
Sockalingum *et al.* 1998	*Pseudomonas aeruginosa, E. coli*	medicine/diagnostics
Subramanian *et al.* 2006	spores of *Bacillus amyloliquefaciens, B. sphaericus, Clostridium tyrobutyricum*	food
Subramanian *et al.* 2007	spores of *Bacillus amyloliquefaciens, B. sphaericus, Clostridium tyrobutyricum*	food
Timmins *et al.* 1998	*Saccharomyces cerevisiae*	food
Winder & Goodacre 2004	*Bacillus*	general, environment
Yu & Irudayaraj 2006	*Enterococcus faecium, Salmonella enteritidis, Bacillus cereus, Yersinia enterocolitica, Shigella boydii*	food

Table 2: Base medium and trace elements

Compound	Formula	Amount [g/l]
base medium:		
magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.2
calcium chloride	$CaCL_2 \cdot 2H_2O$	0.07
iron sulfate	$FeSO_4 \cdot 7H_2O$	0.01
EDTA	$C_{10}H_{16}N_2O_8$	0.01
potassium dihydrogen phosphate	KH_2PO_4	0.6
dipotassium hydrogen phosphate	K_2HPO_4	0.9
trace elements:		
manganese sulfate	$MnSO_4 \cdot H_2O$	0.02
boric acid	H_3BO_4	0.01
copper sulfate	$CuSO_4 \cdot 5H_2O$	0.01
ammonium molybdate	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02
zinc sulfate	$ZnSO_4 \cdot 7H_2O$	0.01

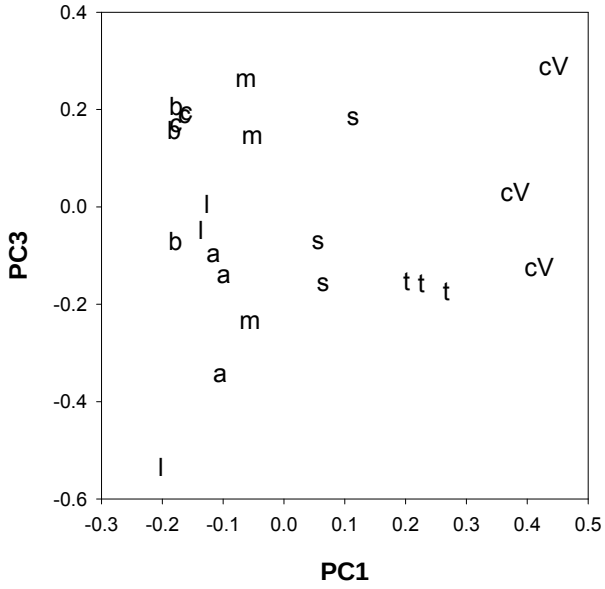
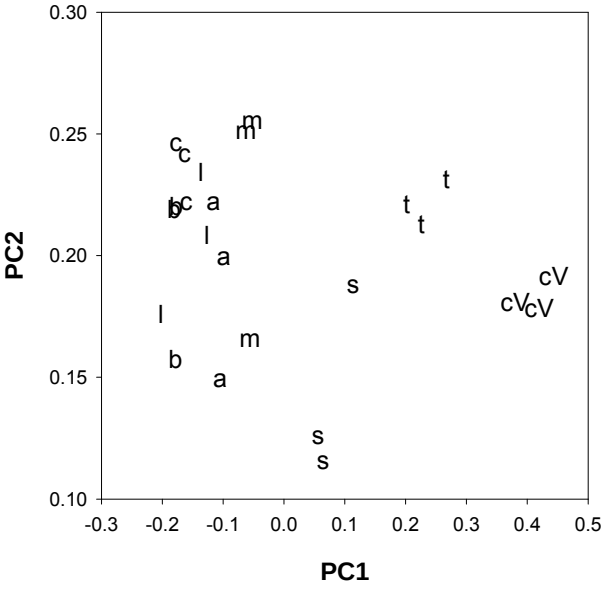
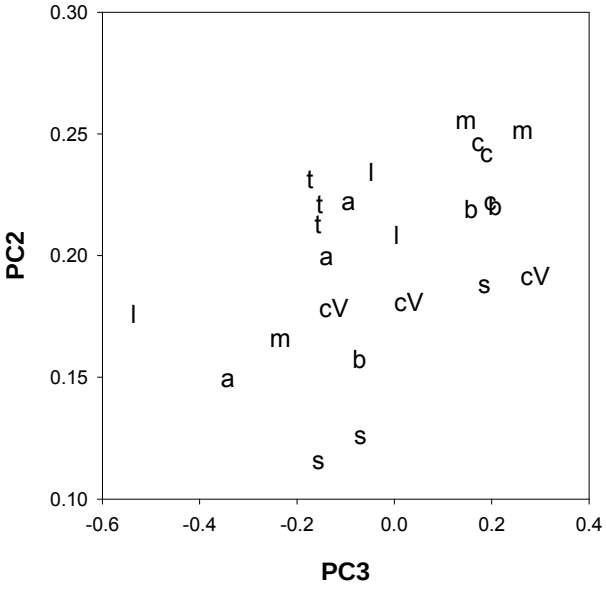


Fig. 1: FTIR-ATR with diamond. First three components (PC1, PC2, PC3) of Principal Components Analysis of the spectral data are shown.

a = *B. atrophaeus*, b = *B. brevis*, c = *B. circulans*, l = *B. lentus*, m = *B. megaterium*, s = *B. subtilis*, t = *B. thuringiensis*, cV = vegetative cells of *B. circulans*





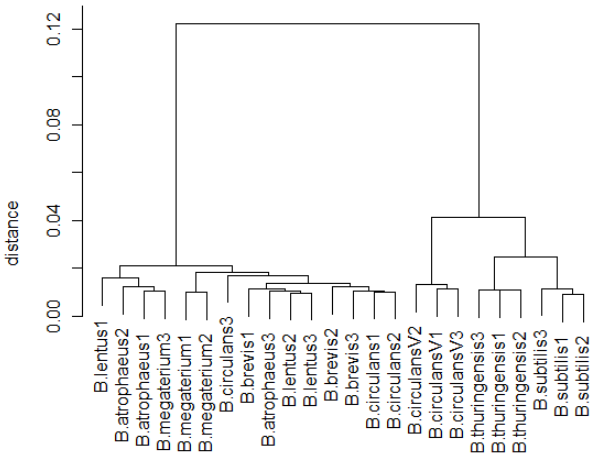


Fig. 2: FTIR-ATR with diamond. Hierarchical cluster analysis (Ward) of pure cultures. The same data as in Fig. 1 are used. V = vegetative cells (*B. circulans*)

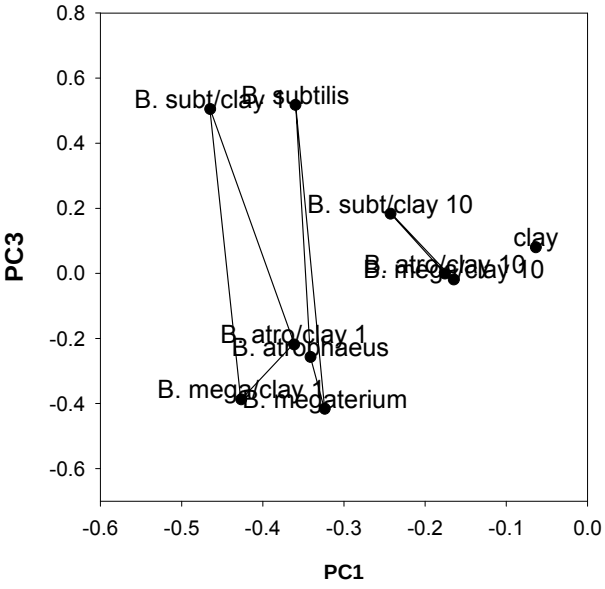
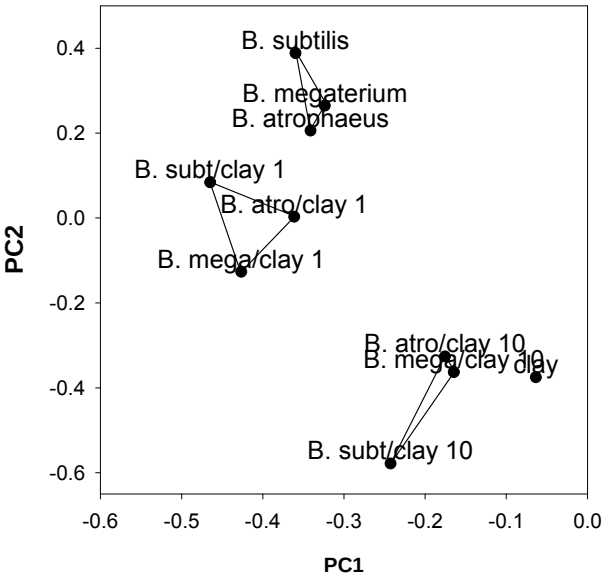
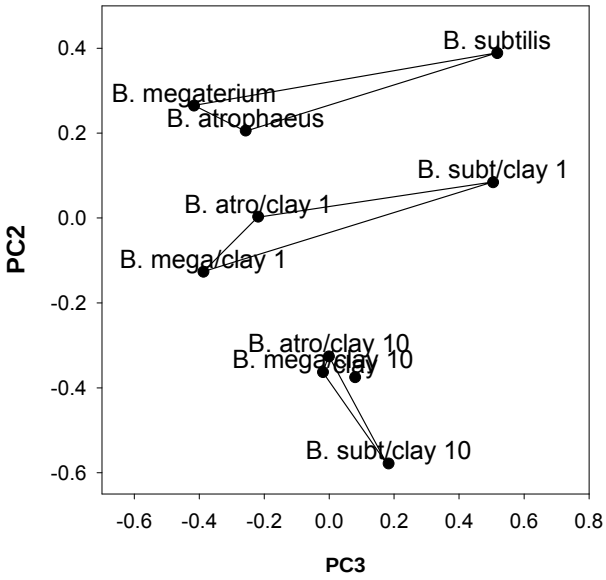


Fig. 3: FTIR-ATR with ZnSe crystal. First three components (PC1, PC2, PC3) of Principal Components Analysis of the spectral data are shown. Pure spore powders and different mixtures with clay (wt/wt) are separated.





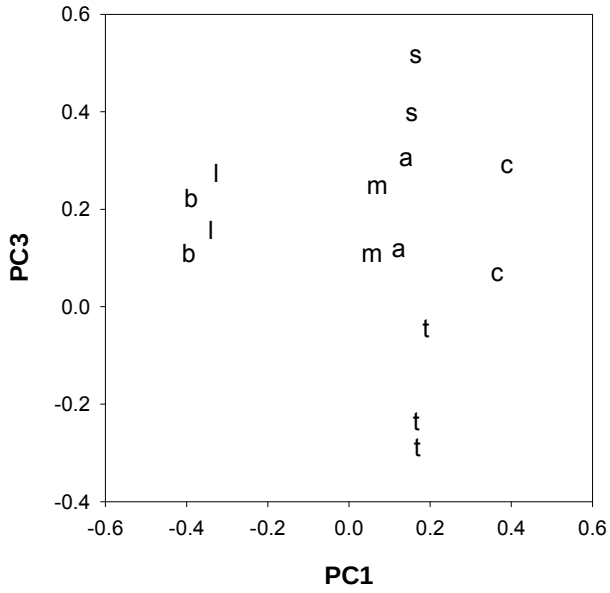
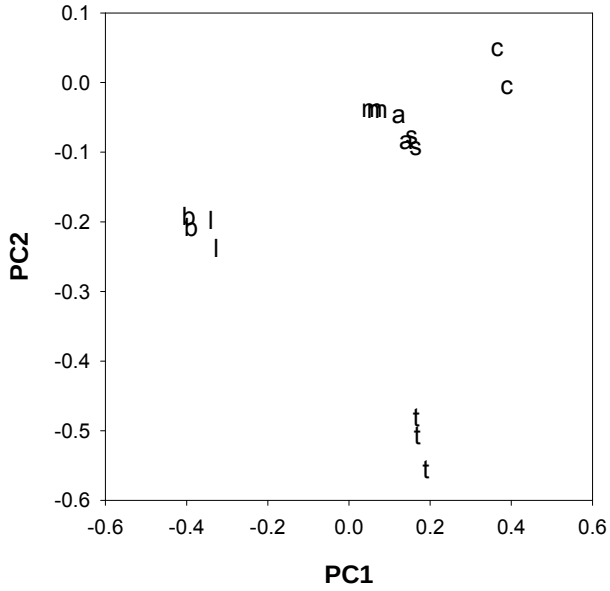
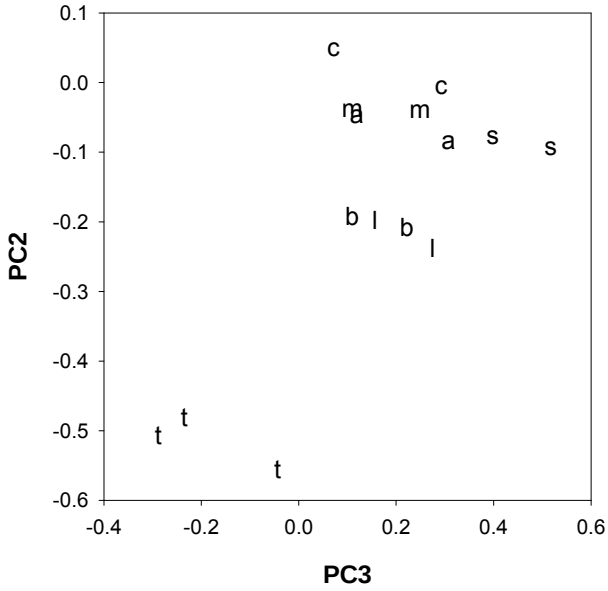


Fig. 4: FTIR transmission. First three components (PC1, PC2, PC3) of Principal Components Analysis of the spectral data are shown. Most species are separated.
a = *B. atrophaeus*, b = *B. brevis*, c = *B. circulans*, l = *B. lentus*, m = *B. megaterium*, s = *B. subtilis*, t = *B. thuringiensis*





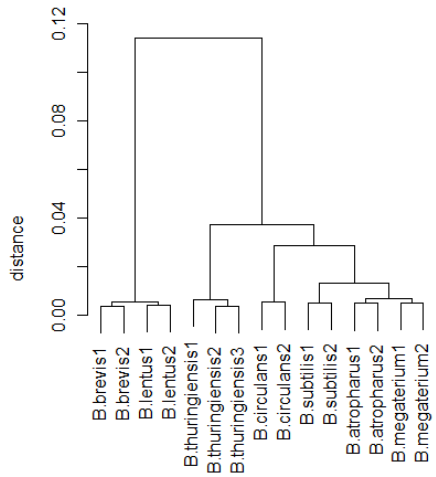


Fig. 5: FTIR transmission. Hierarchical cluster analysis (Ward) of pure cultures. Same data used as in Fig. 4.

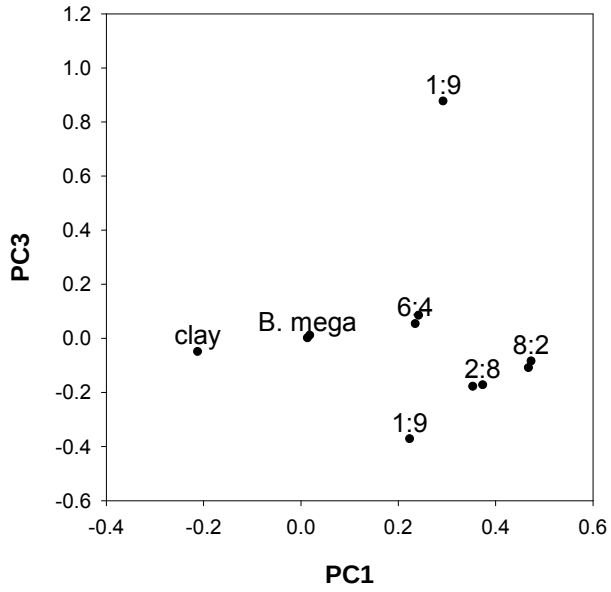
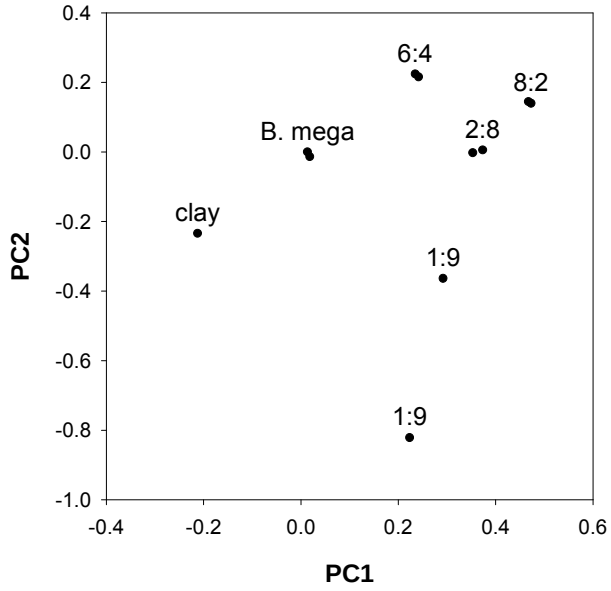
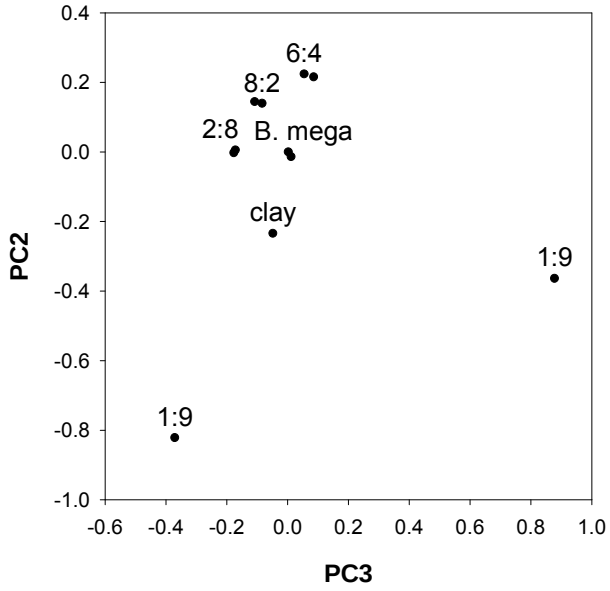


Fig. 6: FTIR transmission. First three components (PC1, PC2, PC3) of Principal Components Analysis of the spectral data are shown. Pure spore powder of *B. megeterium* and different mixtures with clay (wt/wt; 1:9, 2:8, 6:4, 8:2) are separated.





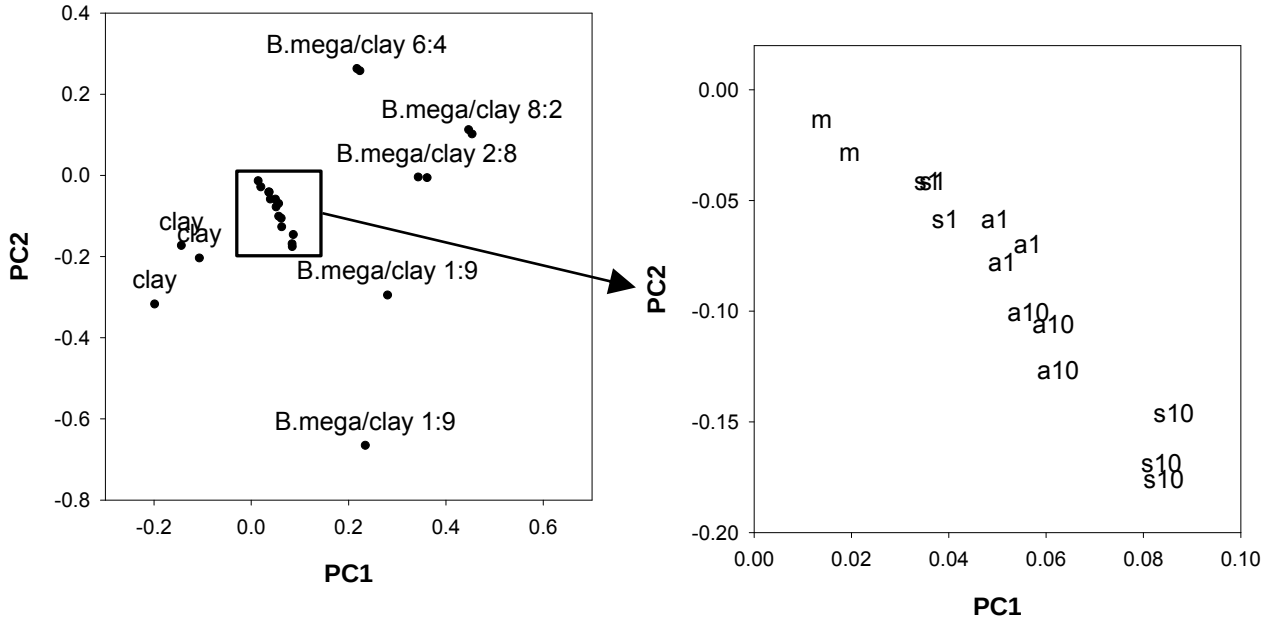


Fig. 7: FTIR transmission. First two components (PC1, PC2) of Principal Components Analysis of the spectral data are shown. *Bacillus* spores and different mixtures with clay (wt/wt).
a1 = *B. atrophaeus*/clay 1:1, a10 = *B. atrophaeus*/clay 1:10, m = *B. megaterium*, s1 = *B. subtilis*/clay 1:1, s10 = *B. subtilis*/clay 1:10

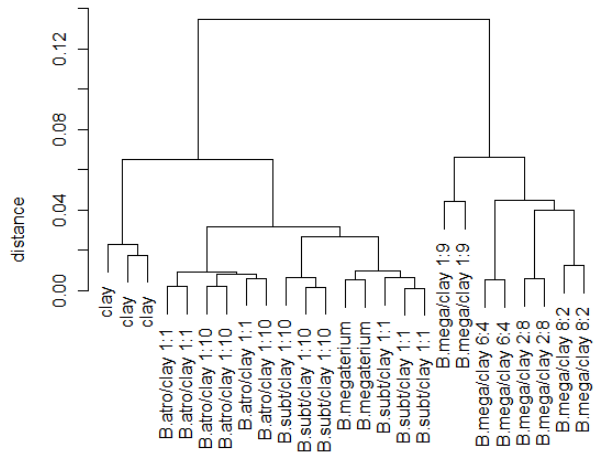


Fig. 8: FTIR transmission. Hierarchical cluster analysis (Ward) of different *Bacillus* spores and different mixtures with clay (wt/wt). Same data used as in Fig. 7.

Chapter III

Detection and identification of *Bacillus anthracis* in soil by cultivation

The use of selective media PLET (polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate) and ACA anthracis chromogenic agar) for the detection of *Bacillus anthracis* in soil samples

Abstract

Bacillus anthracis is a pathogenic organism classified in risk group 3 and thus has to be handled under biosafety level (BSL) 3 conditions. However, the detection and identification with selective agar might be done in BSL 2 if the incubated plates are not opened for inspection. We tested the use of PLET (polymyxin, lysozyme, ethylenediaminetetraacetic acid, thallium acetate) agar and R&F ACA (anthracis chromogenic agar) with soil from a former carcass burial site possibly contaminated with *B. anthracis*. In principle, selective media are applicable for this kind of samples. For the suspicious site, no *B. anthracis* contamination could be verified.

Introduction

Bacillus anthracis is the causative agent of anthrax, an infectious disease, with lethal potential for humans and animals. It is a pathogenic organism classified in risk group 3 and thus has to be handled under biosafety level (BSL) 3 conditions. This means a double containment in a safety laboratory with airlock entry, negative pressure inside, HEPA (high efficiency particulate air) filters for exhaust in air supply system and more. All these safety measurements limit the institutions which are allowed to work with organisms of risk group 3, even for detection only. In some countries it is even more complicated to work with *B. anthracis*, because it is a bioterrorism agent. In the US for example you are registered personally and your reputation gets checked. Our aim is to find safe and easy methods to screen environmental samples for anthrax, which can be applied with less stringent biosafety requirements, if there is a minor suspicion only.
The detection and identification of specific organisms with selective and chromogenic agars is one possibility and might be done in BSL 2 in a biological safety cabinet if the incubated plates are not opened for inspection. We tested self made and commercially available agar plates for use with soil samples.

Materials and Methods

PLET agar: PLET agar is a selective medium, developed by Knisely (1966) and recommended by the WHO for determining *B. anthracis*. As selective agents it contains polymyxin B, lysozyme, EDTA and thallium acetate which inhibit many organisms but allows growth of *B. anthracis*. For detailed composition information see Table 1.

Anthracis chromogenic agar, ACA: ACA was developed by Juergensmeyer and co-workers (Juergensmeyer *et al.* 2006) in collaboration with R & F Laboratories Downers Grove, Illinois, USA. Two different selection methods are integrated: growth inhibition and coloration of colonies. The basic principle of chromogenic media is based on identification by color of the colony. In ACA the coloration derives from the phosphatidylcholine-specific phospholipase C (PC-PLC) activity, an enzyme which is produced only by *B. anthracis*, *B. cereus*, and *B. thuringiensis*. PC-PLC is able to convert a supplement in the medium (5-bromo-4-chloro-3-indoxyl-cholinphosphat, X-CP) into a water-insoluble blue dye (5,5'-dibromo-4,4'-dichloro indigo). In *B. anthracis* the PC-PLC activity is reduced due to a mutation. For this reason the blue color appears later than in colonies of the related species (Fig. 1). Colonies of bacteria without PC-PLC but which are able to grow on the medium appear cream-colored without any touch of blue. For detailed composition information see Table 2.

Preparation of agar-plates: PLET agar with anthracis selective supplement was purchased from Fluka (Sigma-Aldrich), Buchs, Switzerland, prepared according to manufacturer's instructions and plates (Ø 6 cm) were poured in a biological safety cabinet. Chromogenic agar plates (Ø 6 cm) were prepared with PLET as basal medium and 5-bromo-4-chloro-3-indoxyl-cholinphosphat (X-CP) as another supplement (PLET-ACA). The chomogenic supplement was purchased from Biosynth AG, Staad, Switzerland. For an additional experiment ready-to-use ACA plates (Ø 9 cm) from R & F Laboratories, Downers Grove, Illinois, USA were purchased.

Sampling site and sampling: Sampling was conducted at a former carcass disposal site in the surroundings of Zurich, which is nowadays used as riding ring. The last anthrax carcass was buried in 1907. In 1913 spores of *B. anthracis* could still be detected (v. Gonzenbach 1915; Ziegler 2004).

Soil samples were collected in November. Temperature was about 4-6°C, at sunny weather but rain the day before. Sampling was carried out with a soil corer (Ø 15 mm), which was sterilized by flaming in ethanol before each sampling. Cores with a maximum length of 25 cm were obtained, cut in sections of 5 cm and transferred to sterile screw cap PP tubes (20 ml). After return to the laboratory, samples were immediately stored at -80°C until further processing.

Sample preparation: 0.5 g of soil was suspended in 5 ml ultrapure water (18.2 mΩ) in a 15 ml PP tube and vigorously shaken for 3 minutes. To kill vegetative cells, the suspension was heated to 93-97°C for 15 minutes.

Inoculation, incubation and control of plates: 30 µl (6 cm-plates) or 50 µl (9 cm-plates) of the soil suspension, respectively were spread on the plates with a Drigalski spatula, each sample in duplicate. As positive control, a loop full of a non-pathogenic *B. anthracis* strain (kindly provided by Prof. R. Zbinden, Institute of Medical Microbiology, University of Zurich) was streaked onto the agar. In addition, for comparison two PLET and two self-made ACA plates were inoculated with *B. subtilis*. Inoculated plates were incubated upside down at 36°C and checked after 24 and 48 hours for growth and color. After 24 hours, all cream-colored colonies were circled with a marking pen on the back of the plate. After 48 hours, marked colonies were checked for change of color towards blue, the criterion for *B. anthracis*. If new cream-colored colonies appeared, they were marked with a color other than the first ones and plates were checked for a third time after additional 24 hours. In addition, all purchased ACA-plates were photographed at each control to double-check and verify changes in coloration of the colonies.

Results

PLET agar: *B. subtilis* and non-pathogenic positive control for *B. anthracis* did not grow. Bacterial growth occurred from the soil suspensions, but no *B. anthracis* colonies could be identified. Of the 58 plates (29 samples x 2), eleven showed no growth. On most of the plates one, two, three or seven colonies grew, maximum was eleven colonies. For details see Fig. 2. Number of colonies tend to decrease with increasing sampling depth (Fig. 3).

PLET-ACA, self-made: *B. subtilis* did not grow and non-pathogenic positive control for *B. anthracis* did not grow or colonies did not turn blue. From the soil suspensions bacterial growth occurred, but again no *B. anthracis* colonies could be identified. Of the 58 plates (29 samples x 2), seven showed no growth. On ten plates one or three colonies grew, maximum was thirteen. For details see Fig. 2.

ACA, R & F Laboratories: Positive control for *B. anthracis* grew and colonies showed the blue-colored center as described by the manufacturer. From the soil suspensions bacterial growth occurred, too. Many plates were nearly overgrown. No *B. anthracis* colonies could be identified. Although on three of the 112 plates in total (56 samples x 2) a colony with undefined coloration occurred. These samples were sent to another laboratory to test for *B. anthracis* with a polymerase chain reaction method, however, with negative result. The three uncertain samples all originated from a depth between 10 and 20 cm.

Discussion / Conclusions

The use of selective media is a common and relatively easy method to isolate and identify microorganisms. PLET agar has been used successfully for years and thus is recommended by the WHO for the determination of anthrax. ACA is relatively new, introduced at the ASM Biodefense and Emerging Diseases Research Meeting 2003. Several research has been done to test these media with different *Bacillus* species and strains in different matrices (Knisely 1966; Hanes *et al.* 2004; Luna *et al.* 2005; Juergensmeyer *et al.* 2006). Most published results are satisfactory and authors recommend these media for use with food and environmental samples. In a first step, half of the soil samples only were tested with our self-made plates. Regarding the control plates (*B. anthracis, B. subtilis*) results were not as expected. Thus, determination of the colonies from the soil samples was not reliable. Hanes *et al.* (2004) also reported some problems with the identification of *B. anthracis* on PLET but not on ACA. Furthermore, specific strains of *B. cereus, B. megaterium, B. subtilis* and *B. thuringiensis* are reported not to grow on PLET (Knisely 1966). This could explain the poor or lacking growth on our plates, as all contained PLET. To circumvent potential biases regarding self-made agar plates, we purchased ACA plates from R & F Laboratories for a second screening of all the samples. The original ACA seemed to be less selective. In contrast to the modified ACA with PLET as basal medium, many of the plates showed massive growth with only 25% more inoculum. But no colony could be determined as *B. anthracis*. As the carcass had been buried in a depth of about 4 meters and we do not know the exact burial sites, this findings do not mean, that there are no more *B. anthracis* spores at all in the soil of the sampled site. It can be concluded that there is no immediate danger to be infected with anthrax on this site without excavating it.

Advantages of the use of selective media are: 1) there is only minor sample preparation needed; 2) BSL 2 conditions are sufficient, as long as there is no enrichment of the bacteria in a sample before inoculation, no further procession of the grown colonies and the plates stay closed after inoculation, especially if suspicious colonies are grown. But there are also some disadvantages to be mentioned: 1) as it is a culturing method it takes at least 24 to 48 hours until first results are available; 2) determination of organisms based on morphology or visual features of the colony, such as shape and color, need some experience, especially in critical cases; 3) preparation of plates is critical and pre-made plates have a limited shelf-life of 30 days.

Acknowledgements

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Tables & Figures

Table 1: PLET agar

Compound	Amount [g/l]
base medium:	
beef heart infusion (extract from 500 g)	25.00
EDTA	0.30
sodium chloride	5.00
thallous acetate	0.04
tryptose	10.00
agar	15.00
supplement:	
lysozyme	300’000 units
polymyxin B sulfate	100’000 units

Table 2: Anthracis chromogenic agar

Compound	Amount [g/l]
base medium:	
casein digest	15.00
lab lemco powder	5.00
soytone	5.00
sodium pyruvate	10.00
Tween 80 (polyoxyethylenesorbitan monooleate)	0.50
sodium chloride	5.00
manganese chloride tetrahydrate	1.00
cycloheximide	0.20
lithium chloride	2.00
agar	15.00
supplement:	
bovine serum 82-067	3.20
ceftazidime pentahydrate	0.04
5-bromo-4-chloro-3 indoxyl-choline phosphate	0.32
polymyxin B sulfate	100’000 units

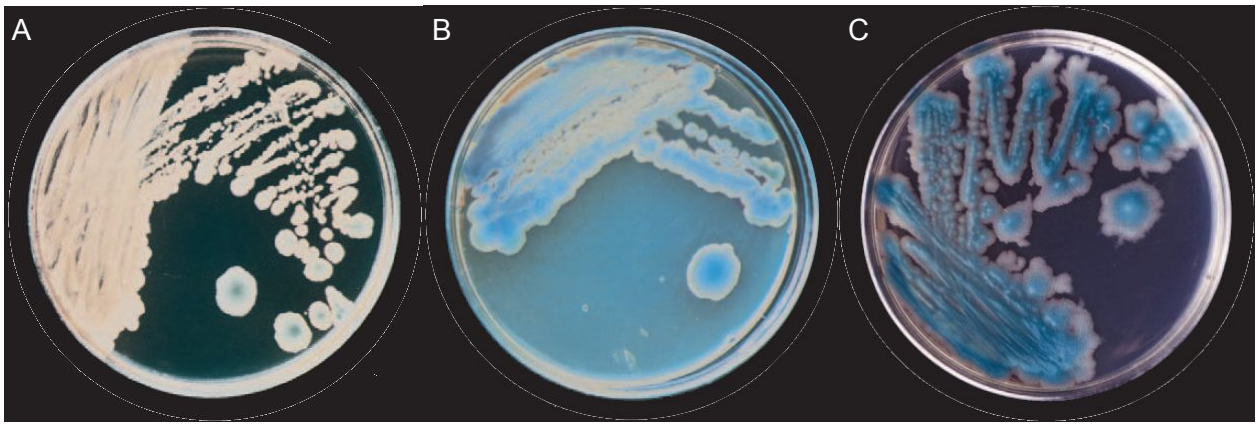

Fig. 1: Anthracis chromogenic agar plates. A: *B. anthracis* after 24 hours; B: *B. anthracis* after 48 hours; C: *B. cereus/B. thuringiensis* after 24 hours. (R&F Laboratories)

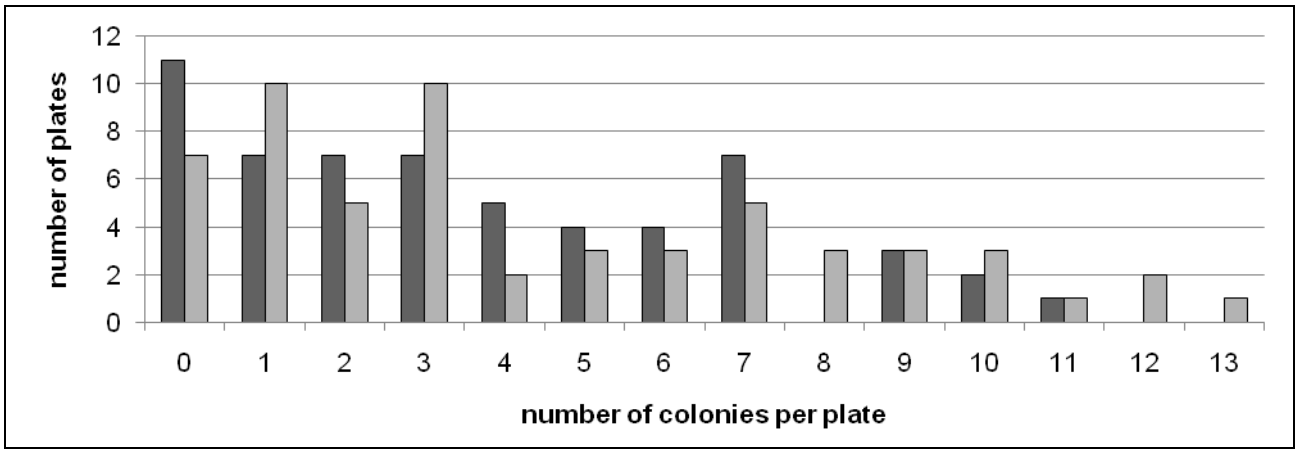

Fig. 2: Number of colonies per plate. Dark bars represent PLET agar, light bars PLET-ACA.

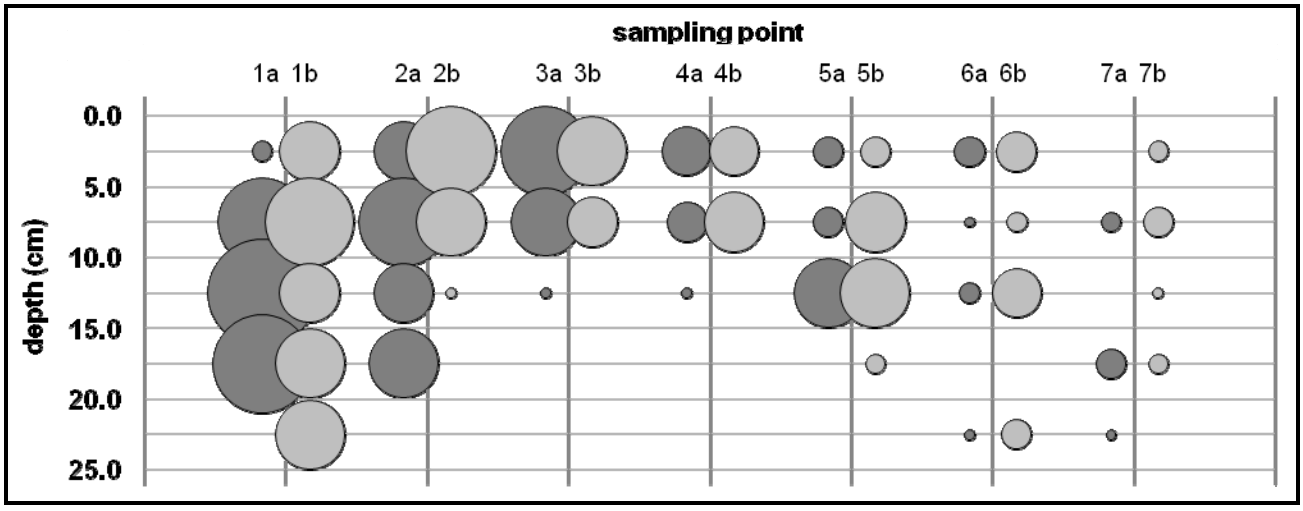

Fig. 3: Number of colonies related to sampling depth. Dark/light points represent replicates, diameter is proportional to the number of colonies.

Chapter IV

Assessment of *Bacillus* diversity in soil by molecular methods

Diversity of *Bacillus* species in soil along a transect and at different depths

Abstract

Bacillus community structure and diversity in soil were analyzed using polymerase chain reaction and temporal temperature gradient electrophoresis, PCR-TTGE fingerprinting technique and a *Bacillus*-specific primer pair for 16S rDNA. Samples were collected from a grassland site along a transect and at different depths. A total of 18 distinguishable bands was found. The community structure was significantly dependent on the sampling position along the transect ($P<0.001$) and to a minor extent by the content of soil organic carbon ($P<0.01$). Sampling depth showed no significance.

Introduction

The genus *Bacillus* belongs to the phylum *firmicutes* (class: *bacilli*, order: *bacillales*, family: *bacillaceae*) and comprises gram-positive, aerobic, endospore-forming bacteria. *Bacillus* species are ubiquitous, quite diverse, due to their ability to build spores extremely resistant to environmental stress. They can therefore survive for many decades or centuries. Bacilli are part of the soil microbial community. Most of spore-forming bacteria are harmless to humans and animals, but some (e.g. *Bacillus anthracis*) can cause severe diseases such as anthrax. In contrast to many laboratory studies, less is known on the occurrence, frequency, and diversity of *Bacillus* species including their endospores in their natural soil environment. The aim of this study was to investigate the diversity and distribution of *Bacillus* species in soil along a transect of a meadow and in different sampling depth and the relationship of soil parameters (organic carbon) and the occurrence of different species. Different types of methods were applied to analyze bacteria in soil. Data presented here were obtained using molecular methods. Results from physico-chemical methods (terbium fluorescence, fourier transform infrared spectroscopy) are presented in chapter II of this thesis. One molecular method often used to explore bacterial communities is the comparison of specific 16S rDNA or 16S rRNA sequences. rDNA encodes for the ribosomal RNA (rRNA). Ribosomes are the protein factories of a cells. They consist of two subunits, a large and a small one. The 16S rRNA is part of the small subunit of procaryotic ribosomes. rDNA was found to be most conserved (least variable) DNA part in all cells. For this reason it is a excellent tool for phylogenetic studies or for the identification of organisms. For community studies often general primers for bacteria are used and the derived sequences have to be further processed to obtain more detailed information. Goto *et al.* (2000) searched and compared the 16S rDNA sequences of 69 *Bacillus* type strains and detected a hypervariant region (HV region) which was highly specific

for each type strain and furthermore highly conserved within species. We used this specific HV region combined with the PCR-TTGE fingerprinting technique to study the *Bacillus* community and diversity in soil.

Materials and Methods

Sampling site and sampling: Sampling site was a former carcass disposal site in the surroundings of Zurich, which is nowadays used as riding ring and for show jumping (for more information see appendix of this thesis). Soil samples were collected in November. Ambient temperature was about 4-6°C, at sunny weather, but rain the day before. Sampling points are located on a 100 m transect (every 10 m) and on a 15 m line rectangular to it and parallel to the edge of a forest (every 5 m), forming a T- shaped sampling scheme (Fig. 1). Sampling was carried out using a stainless steel soil corer (Ø 15 mm), which was sterilized by flaming with ethanol before each sampling. Cores with a maximum length of 25 cm were obtained, cut in sections of 5 cm and transferred to sterile screw cap PP tubes (20 ml). After return to the laboratory, samples were immediately stored at -80°C until further processing.

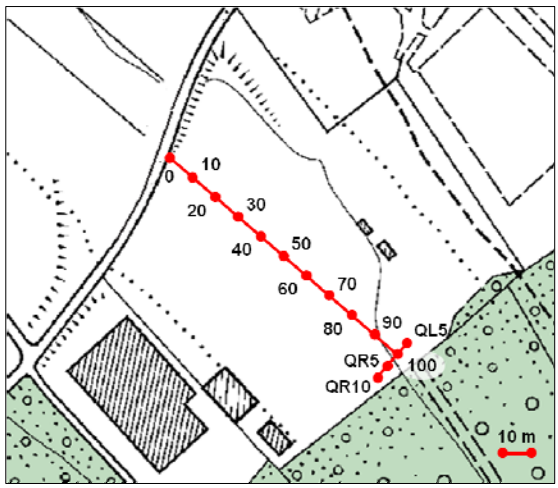

Fig. 1: Sampling site and sampling points. At each point soil cores were taken to a maximal depth of 25 cm. Cores were devided into samples of 5 cm.

DNA-Extraction: Whole DNA was extracted directly from the soil samples using a kit (UltraClean soil DNA isolation kit; MO BIO Laboratories, Inc., Carlsbad, California, USA). 0.5 g of soil was processed according to manufacturer's protocol. To improve access to DNA from endospores, the original protocol was slightly modified upon the manufacturer's recommendation by inserting three freeze-thaw cycles (30 min at -20°C / 15 min at 37°C) after addition of the SDS-solution (S1). As second modification, instead of a flat-bed vortex pad, a TissueLyser Qiagen (Retsch GmbH & Co, Haan, Germany) was used for bead beating. Extracted DNA was stored at -20°C until further processing.

Primers: Primers for a *Bacillus*-specific 16S rDNA sequence, the hypervariant region (HV) were used. They were designed by Goto and co-workers (Goto *et al.* 2000) and are described to be even highly specific for strains. For our purpose one primer was prolonged with a GC-rich sequence at the 5' end. This GC-clamp prevents PCR products from complete melting during separation by gradient electrophoresis and thus improves the resolution of separation (Sheffield *et al.* 1989). The best primer combination was determined in pre-experiments (data not shown): forward primer with CG-clamp

(5'-*CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG*-TGTAAAACGACGGCCAGTGCCTAATACATGCAAGTCGAGCG-3') and original reverse primer (5'-CAGGAAACAGCTATGACCACTGCTGCCTCCCGTAGGAGT-3'). Primers were synthesized by Microsynth AG, Balgach, Switzerland.

PCR: For polymerase chain reaction (PCR) we used Qiagen HotStarTaq (Qiagen AG, Hombrechtikon, Switzerland). The PCR mixture (25 µl) consisted of 12.5 µl HotStarTaq Master Mix (= 2.5 U Taq, 0.2 mM dNTP), 0.5 µM of each primer, 1 µl (ca. 19-74 ng/µl) template DNA and H_2O. The reaction was performed with a TGradient thermocycler (Biometra GmbH, Goettingen, Germany) according to the following protocol determined by pre-experiments: denaturation for 15 min at 95°C, 35 cycles including 45 sec at 94°C, 45 sec at 62.5°C and 1 min at 72°C, followed by a final extension for 10 min at 72°C and cooling at 4°C. Concentration of template DNA was measured spectroscopically with a plate reader (SpectraMax M2, Bucher Biotec AG, Basel, Switzerland) using Hoechst 33258 fluorescent dye (Pharmacia/GE Healthcare AG, Glattbrugg, Switzerland, 300 ng/ml 1xTNE [tris(hydroxymethyl)aminomethane - NaCl - ethylenediaminetetraacetic acid]). Base pair length of PCR products was checked by agarose gel electrophoresis.

TTGE: Temporal temperature gradient electrophoresis (TTGE) was performed with a DCode System (Bio-Rad Laboratories AG, Rheinach, Switzerland) according to the instruction manual. Gels were of 1 mm thickness and composed of 8% (wt/vol) acrylamide–N,N'-methylenebisacrylamide (37.5:1), 1x TAE buffer (tris(hydroxymethyl)aminomethane - acetate - ethylenediaminetetraacetic acid), 8 M urea, 20% (vol/vol) formamide, 0.1%(wt/vol) ammonium persulfate and 0.1% (vol/vol) TEMED (N,N,N',N'-tetramethylenediamine). In modification from the Bio-Rad protocol we added formamide to the gel for better denaturation (Heuer *et al.* 1997) and we used 1x TAE instead of 1.25. Samples were mixed with loading dye (4 µl + 4 µl) and loaded with gel loading tips. Gels were run at 130 V, in the temperature range of 60-70°C and a temperature ramp of 1.2°C/h. Temperature range was determined with Poland's algorithm (Steger 1994) online on http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html.

Determination of organic carbon: Organic carbon was determined by weight loss after combustion. Aliquots of the soil samples were dried at 110 C for 10 hours in a drying oven (WTC Binder GmbH, Tuttlingen, Germany). Approximatively 1 g of dried soil was weighed into a titanium crucible and heated at 550°C for 2 hours in a muffle furnace (L 47 T, Nabertherm GmbH, Lilienthal, Germany). Samples were subsequently cooled in a desiccator for weighing. The weight loss corresponds to the amount of organic carbon in a sample.

Gel analysis and statistics: Pictures of the TTGE gels were processed and band patterns analyzed with the software package GelCompar II (Applied Maths NV, Sint-Martens-Latem, Belgium) according to the provider's instructions. Diversity with respect to different sample groups

was analyzed performing Principal Components Analysis (PCA) and Multi-Dimensional Scaling (MDS) on the band matching table. Statistical analysis was done using the open source software package R (R Development Core Team 2009). The influence of different factors (sampling point [x = long axis, y = short axis], sampling depth, organic carbon) on the *Bacillus* diversity (number of bands) was tested with a linear model and ANOVA. Where applicable, data were transformed for better fit (formulas mentioned in results). Dependency of the content of organic carbon on sampling point and depth was analyzed and tested the same way.

Results

Organic carbon: The content of organic carbon ranged from 4.9 to 18.4 percent. It was significantly dependent on the sampling depth ($P<0.0001$) and on the sampling position ($P<0.01$, details see Table 1). Carbon content was decreasing with depth and tended to be higher on the edges of the site (Fig. 2).

Total DNA: The total DNA yield per sample differed significantly depending on sampling position ($P<0.0001$), depth ($P=0.05$) and content of organic carbon ($P=0.01$). DNA yield tended to increase with increasing distance to the forest and there was an increase down the slope along the forest, too (the most elevated point is nearest the forest). Depth was slightly negatively correlated, organic carbon positively (log) (Fig. 3). There was a significant negative correlation ($P<0.0001$) between number of bands and total DNA yield (Fig. 4).

Diversity: 18 different *Bacillus* species or bands were found (see appendix of this thesis for band matching table). In most samples (62.5 %) only one species was present, in some no or 5 – 7 and in one sample a maximum of 14 species. For detailed information see Fig. 5.
PCA and MDS of the band matching table revealed no clustering regarding sampling point, sampling depth or content of organic carbon (see appendix of this thesis). Nevertheless ANOVA with χ^2 test showed that the number of bands is significantly influenced by the sampling position ($P<0.001$) and to a minor extent by the content of organic carbon ($P<0.01$ details see Table 2). Sampling depth shows no significance. The model with the best fit (smallest Akaike information criterion, AIC) is a generalized linear model (glm) with Poisson distribution:

$$\text{bands} \sim \text{pos x} + (\text{pos x})^2 + (\text{pos x})^3 + \text{pos y} + \text{factor(POS)} + \text{org C} + \text{pos x:org C} + \text{pos y:org C}$$

The correlation graph of number of bands and sampling position shows a tendency of higher diversity at the edges of the site, particularly near the forest (Fig. 6A). There it is positively correlated with the slope of the terrain. Corresponding graphs of the content of organic carbon look similar (Fig. 2B), but the direct correlation of number of bands and content of organic carbon is slightly negative (Fig. 6B).

Discussion / Conclusions

The decrease of organic carbon with depth is obvious because of the decreasing amount of roots and other plant material or other influences from the surface, such as input from detritus and rain. Total number of species is comparable to the findings of Garbeva *et al.* (2003) who compared *Bacillus* species in soils of grassland and arable land.

Diversity was expected to be more dependent on and positively correlated with the amount of organic carbon, because of less competition. If the data of the different sampling positions are pooled (ignoring depth) there is a positive trend in correlation (Fig. 7). Regarding the total extracted DNA the impact of organic carbon might be stronger on other bacteria or on the quantity of some *Bacillus* "main species" than on *Bacillus* diversity, but this study did not include quantitative analysis of bands. Zhou *et al.* (2002) investigated bacterial diversity at different depth in soils with different concentrations of total organic carbon. They found that diversity was significantly influenced by organic carbon. It was lower in high-carbon (1.3 – 75% organic C) soils than in low-carbon (<0.5% organic C) soils. Depth had no influence in high-carbon soils. This is not comparable to our study, because Zhou and co-workers had much broader depth ranges (meter scale), as this is the case in all studies which have a look on depth. Earlier investigations (Vavulo & Karbanovich 1965; Mishustin 1968) relying on culturing and isolation already found the soil type to be the main influence on the composition of a *Bacillus* population, along with vegetation and land management. But independent from soil type most bacteria were found at the surface and not all *Bacillus* species penetrated the soil to an equal depth. This infers that there is also an influence of depth. Siala and co-workers (1974) found an influence of depth on the composition of the community and abundance of species regarding spores. More recent studies on molecular basis (e.g. Torsvik *et al.* 1998; Garbeva *et al.* 2003) show as well a dependency of *Bacillus* diversity on vegetation and/or agricultural treatment. Our sampling site is grassland actually used as riding ring and for show jumping. The tendency of higher *Bacillus* diversity at the edges of the site might be due to higher diversity in vegetation and less disturbance.

In over 60% of the samples only one species was detected. This might be a bias of the method, although PCR with gradient gel electrophoresis emerged to a routinely used technique to study microbial diversity since the 1990ies (Muyzer 1999). An overview of applications of denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE) in microbial ecology is presented in Muyzer & Smalla (1998). Identified limitations were tried to be improved, e.g. by application of species-specific PCR primers and GC-clamp. Total extracted DNA yield was checked, but there might have been a considerable proportion of non-*Bacillus* DNA. And high template DNA concentrations may hamper amplification and result in inadequate PCR products.

Total DNA yield is lower near the forest. This might be explained by the influence of the trees (spruce and fir) on the soil characteristics and microflora. Kuske and colleagues (1998) determined

lower soil DNA yields for forest than for agricultural soil. The negative correlation of total extracted DNA with depth is linked to the content of organic carbon and corresponds to the findings of Agnelli *et al.* (2004) who studied microbial communities at a comparable depth scale.

Studying bacterial diversity in soil is a really challenging task. First, results can be completely different depending on the applied methods. For example Axelrood and co-workers (Axelrood *et al.* 2002a; 2002b) analyzed bacterial diversity of the same samples with culturing or molecular methods. Results differed in community composition in general. Regarding the *Bacillus/Clostridium* group (spore forming bacteria), they were found in all samples with the former method and only in one sample and only to a low percentage with the latter. Second, soils are one of most difficult environmental matrices from which to obtain microbial DNA for adequate PCR (Kuske *et al.* 1998). For example humic acids as soil compounds are potent inhibitors of the reaction. Third, microbial diversity is a question of scale. Especially in soil there are so many microsites with totally different conditions and therefore their own specific microcommunities (Kennedy 1999). According to Nunan *et al.* (2002) microbial biomass was found to be greater in preferential flow paths than in the soil matrix. While homogenizing samples all these differences and thus part of the diversity get lost and diversity patterns become altered. Our study site might show more diversity looking at the micro-scale.

Acknowledgements

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Tables & Figures

Table 1: Analysis of variance table.

Linear model: organic C ~(position x + position y) * log(depth)

	degrees of freedom	**sum of squares**	**mean squares**	**F value**	**Pr(>F)**
position x	1	29.556	29.556	8.8937	0.004486
position y	1	5.375	5.375	1.6175	0.209570
depth (log)	1	301.007	301.007	90.5757	1.248e-12
posx:logd	1	2.236	2.236	0.6727	0.416158
posy:logd	1	12.266	12.266	3.6909	0.060660
Residuals	48	159.516	3.323		

Table 2: Analysis of deviance table (χ^2 test)

General linear model with Poisson distribution:

bands ~ pos x + $(\text{pos x})^2$ + $(\text{pos x})^3$ + pos y + factor(POS) + org C + pos x:org C + pos y:org C

	degrees of freedom	**deviance**	**resid. degrees of freedom**	**resid. deviance**	**P(>|Chi|)**
NULL			53	140.711	
position x	1	11.741	52	128.970	0.001
position y	1	34.273	51	94.697	4.79e-09
factor position	11	28.133	40	66.565	0.003
org. C	1	7.703	39	58.862	0.006
posx:orgC	1	6.368	38	52.494	0.012
posy:orgC	1	4.722	37	47.772	0.030

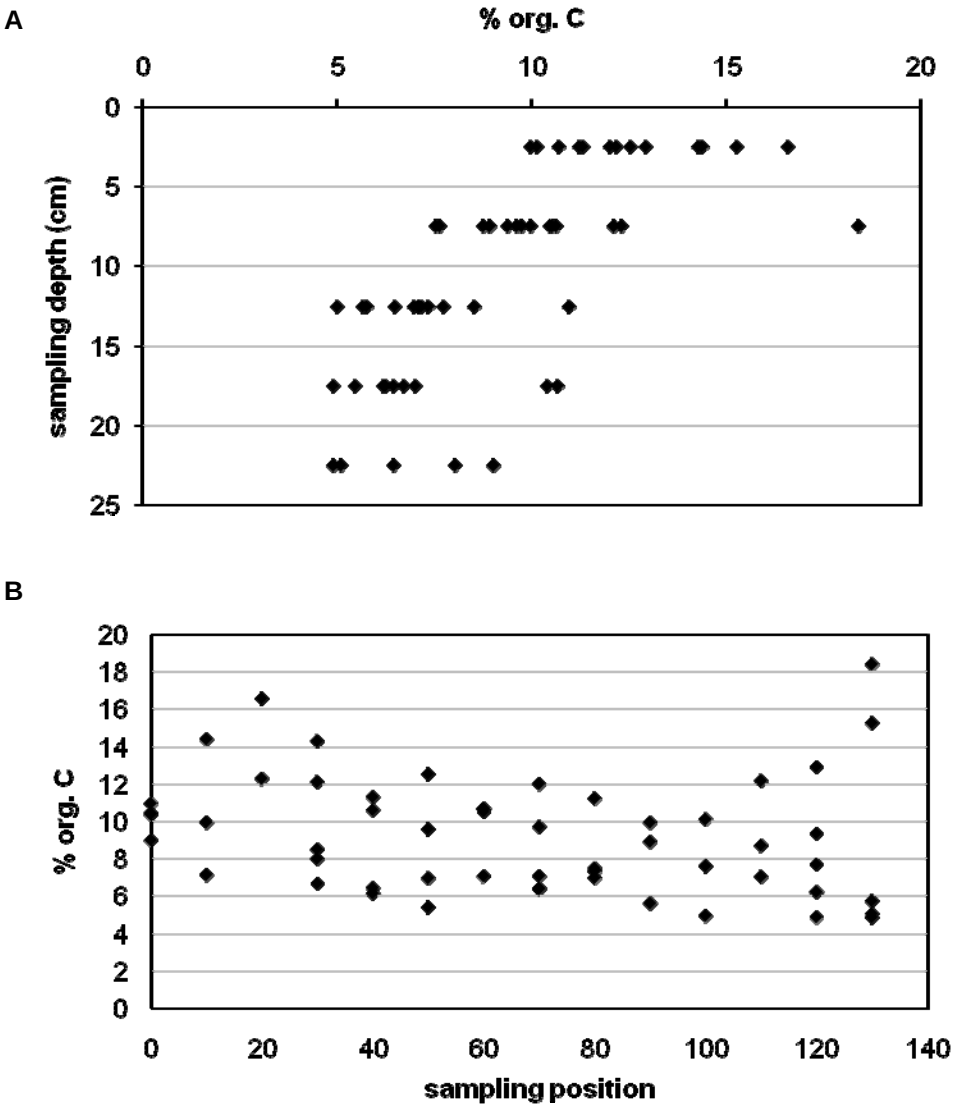

Fig. 2: Correlation of % organic C in soil and sampling depth (A) and sampling position (B; position 0 to 100 on long axis, position 100 to 130 on short axis).

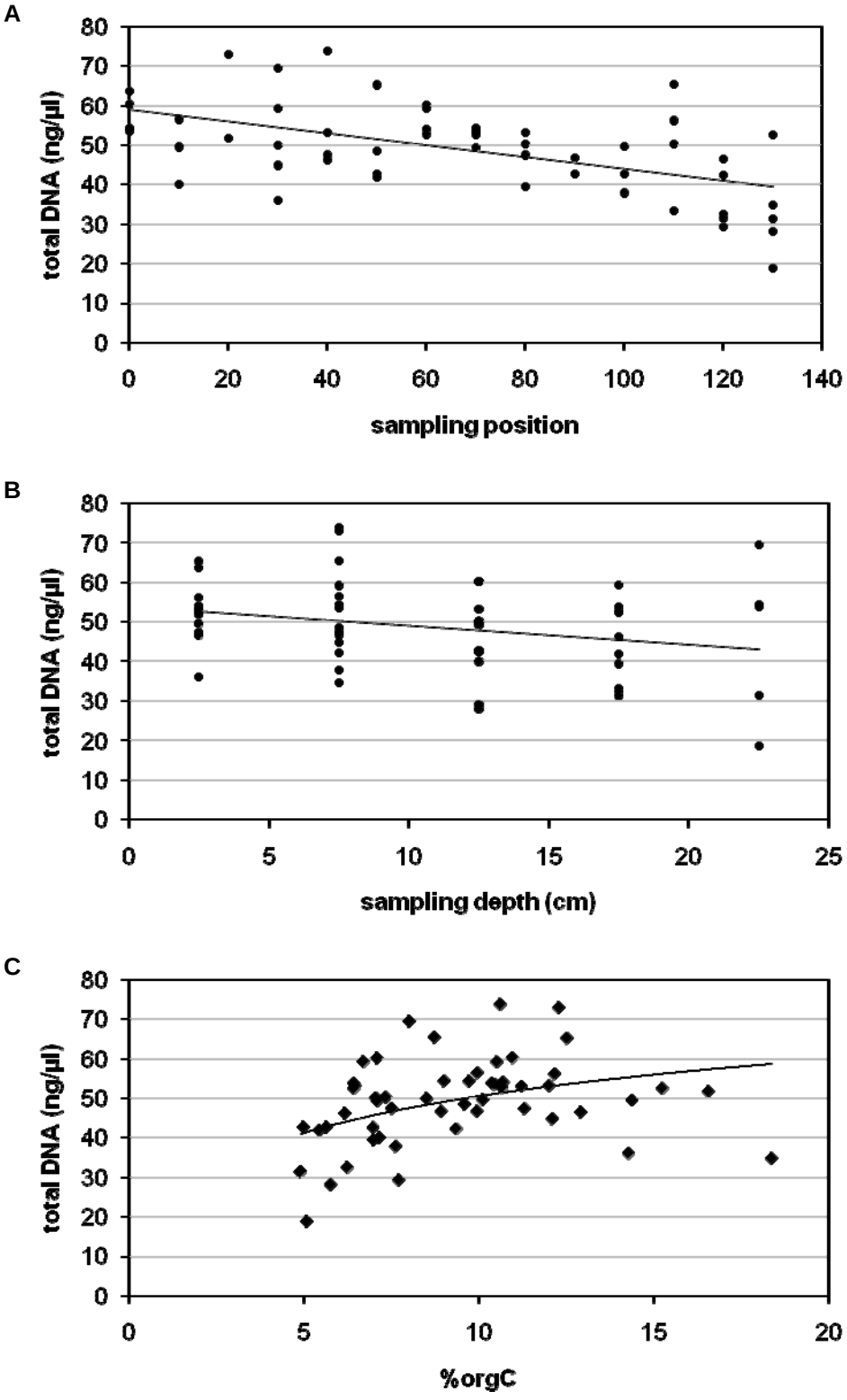

Fig. 3: Correlation of total DNA yield with sampling position (A; position 0 to 100 on long axis, position 100 to 130 on short axis), sampling depth (B) and % organic C in soil (C).

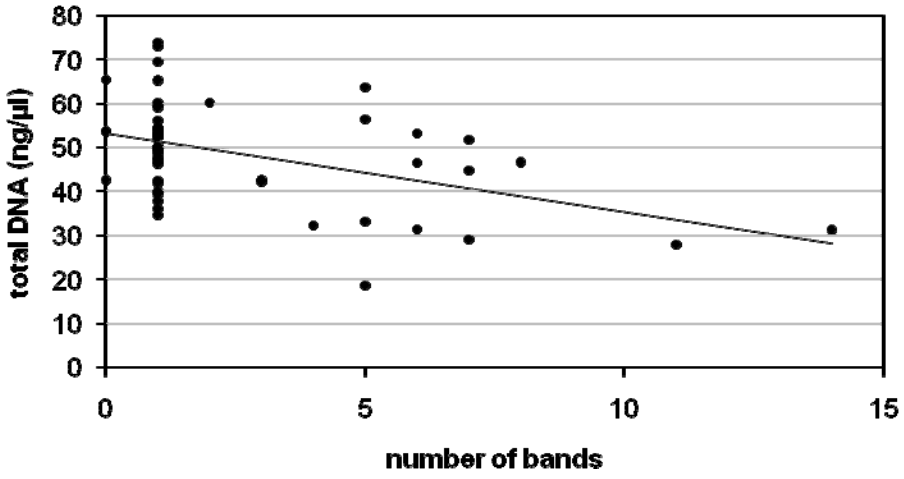

Fig. 4: Correlation of number of bands with total DNA yield.

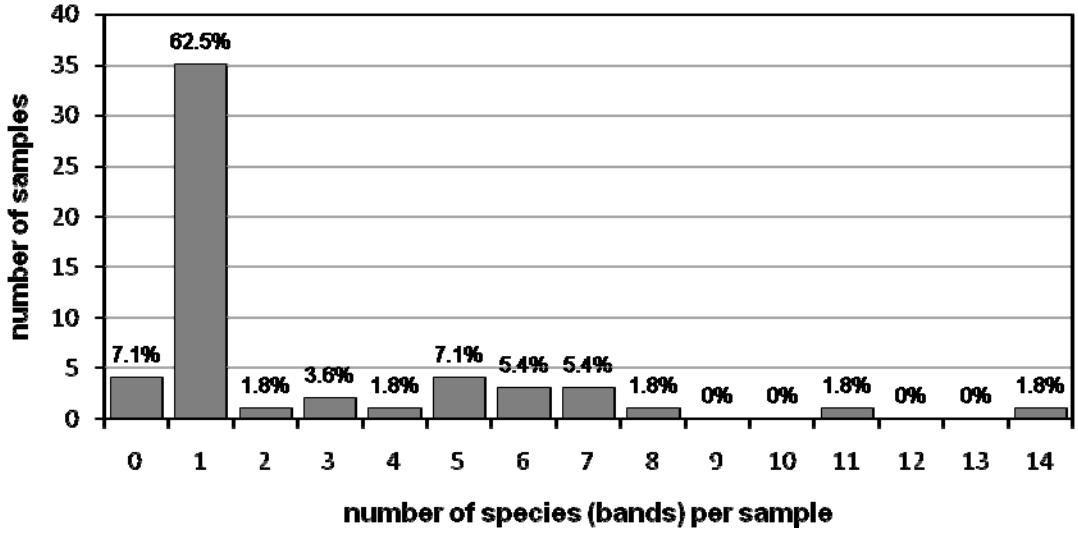

Fig. 5: Distribution of the number of species (bands) per sample. In most samples (62.5%) only one species was found. The maximum was 14 species but in one sample only.

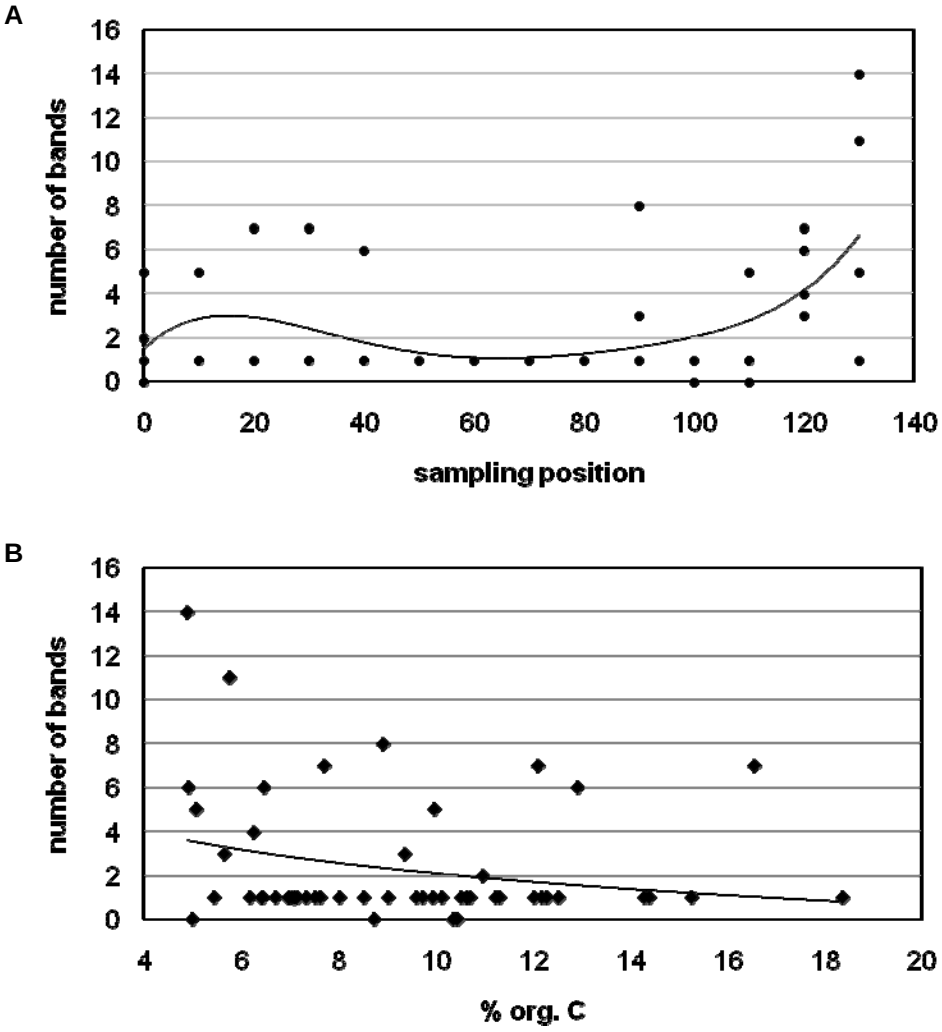

Fig. 6: Correlation of number of bands (species) with sampling position (A; position 0 to 100 on long axis, position 100 to 130 on short axis near the forest) and % organic C in soil (B).

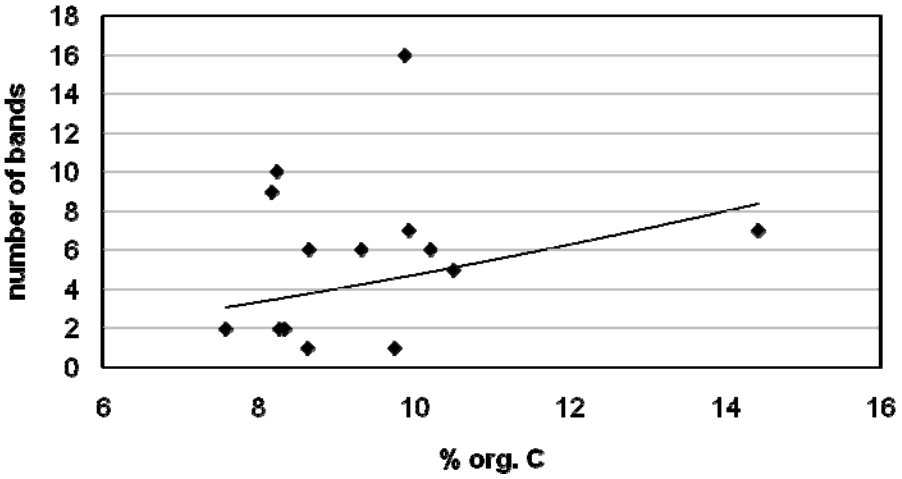

Fig. 7: Correlation of number of bands (species) with % organic C in soil. Data of sampling positions are pooled, ignoring depth. In contrast to Fig. 6B (data not pooled), there is a positive trend in correlation.

Discussion

Discussion

Trigger for this work was the world wide “anthrax crisis”. Mail contaminated by anthrax in the USA in 2001 and the subsequent hoax and threatening letters of copycats all over the word. The public was not only frightened by letters but also by all kinds of somehow suspicious looking powders and similar substances. Hence hundreds of environmental samples had to be collected and analyzed for *Bacillus anthracis*.

As anthrax once was a relatively widespread infectious animal disease, specific locations could still be contaminated with viable spores and serve as natural reservoirs. Therefore the responsible authorities of the canton of Zurich requested to have an overview of these “natural” anthrax sites. This led to the first part of this thesis: The historical investigations about anthrax in the canton of Zurich and the land register of possibly contaminated locations.

Whenever an “anthrax-alarm” is given, police, firefighters and a biological advisor move out to secure the “site of crime” and to seize samples. Because chemical decontamination (mostly with Javelle water, sodium or potassium hypochlorite) may cause damage to objects and environment, sometimes personal goods, rooms, apartments, offices or even whole buildings or infrastructure have to be confiscated and sealed off until it is clear if there was a real threat by anthrax or not. In the beginning relatively much time was required until reliable results from the sample analysis were available. To minimize the time span to the release of confiscated and sealed off items, fast, easy and inexpensive detection methods applicable for environmental samples, and as far as possible for field use were needed. Part of this work was to evaluate and (further) develop such methods. The focus are microorganisms, bacterial endospores in a complex environmental matrix such as soil. The first main task – a more practical one – was how to detect and identify them specifically in a fast, easy and inexpensive way without the need of a highly technical safety laboratory. The second main task – the more scientific one – was related to a specific group of these microorganisms, the genus *Bacillus*, their diversity and distribution. The first task provided the tools for the second one.

Non-invasive physico-chemical methods were first determined to be the most adequate approach for the first task. As a first step a fluorescence method was developed, knowing, that dipicolinic acid (DPA) is a chemical component exclusively present in bacterial spores and that it forms a fluorescent chelate complex with terbium. The strength of the fluorescence signal corresponds to the amount of DPA available in a sample and, to the number of endospores, respectively. As the DPA content is dependent on species and to a certain extent on sporulation conditions an external calibration is needed, for example the standard addition method. The amount of spores per sample is then determined as equivalents of a specific species (e.g. *Bacillus subtilis*). This fluorescence method is easy, relatively fast (about 1-2 h) and inexpensive. Because all bacterial spores contain DPA it is only spore- but not species-specific. This is a disadvantage since the determination of

diversity and specific distribution requires a species-specific method. However, therefore a next step was to work out an infrared (IR) spectroscopic method. The basic principle of this approach is relatively simple. The molecules and atoms in a sample are excited to vibrate and rotate by an infrared beam. The sum of all these movements can be made visible as a absorption spectrum through a mathematical transformation (Fourier transformation, → FTIR). Spectra are dependent on the chemical components and for this reason specific for a sample. By further mathematical procession of the spectra, cluster analysis, dimensioning techniques (Principal Components Analysis, PCA; Multi-Dimensional Scaling, MDS) or comparison to a spectral database it is possible to determine the species or to verify the presence of a specific organism in a sample. As well as the fluorescence method, FTIR is easy, fast (max. 1 h), inexpensive and, in addition, species-specific. Though, there is another drawback instead, strong matrix effects with soil. It turned out, that this method is not applicable for the detection of bacilli and their endospores in soil, but only in simple matrices.

Although this physico-chemical methods are not fully satisfactory for addressing our second task, namely diversity and distribution of *Bacillus* species in soil, they are at least a partial solution for the first. As a primary analysis they give useful information about the presence of endospores (fluorescence) or biological contamination (FTIR). Different portable devices for first responders have been developed applying one of these principles as basic method. Analysis requires only a few minutes. As an example might be mentioned the “anthrax smoke detector” (ASD), described by Lester and Young and co-workers (Lester & Ponce 2002; Lester *et al.* 2004; Yung *et al.* 2007), which was engineered in collaboration with NASA. This detector is for air samples only. Another device is the EDS2000 endospore detection system by Ocean Optics (Dunedin, FL, USA, www.oceanoptics.com). It analyzes swabs from surfaces but is already not commercially available anymore. A product by Veritide Ltd. (Christchurch, New Zealand, www.veritide.com) is based on advanced fluorescence and photo-chemical techniques. The so called Ceeker can detect spores in dry and liquid samples. FTIR technology is used in the HazMatID by Smiths Detection (Watford, Herts, UK, www.smithsdetection.com). The device is mainly a chemical identifier for hazardous chemicals but it can detect biological contamination by the presence of proteins. It is applicable to any kind of sample.

One of the oldest detection and identification methods is culturing and isolating. However, this type of method does not meet the requirements of being fast and applicable for field use. Moreover, it can only be applied to culturable organisms. In addition, depending on the organism it can even be hazardous because of enrichment and sample manipulations. In case of *B. anthracis* normally a laboratory with minimum biosafety level (BSL) 3 is required. Recently developed selective chromogenic agars are one-step culturing methods and the plates do not need to be opened after incubation. This advantage allows a lower biosafety level for the analysis of environmental

samples. Hence selective chromogenic agars may be an option when no BSL 3 laboratory is available.

Protocols have been established for different molecular methods such as polymerase chain reaction (PCR) or fluorescence in-situ hybridisation (FISH). These methods can be species-specific and are relatively fast (some hours). As a disadvantage the established protocols are often not suitable for the use with complex matrices, such as soil samples. Since validated protocols for the detection of biological warfare agents are already available, we did not further pursue molecular methods for this purpose. There even exist PCR based hand-held, fully field portable devices for use by first responders with little experience with biological testing (e.g. Bio-Seeq PLUS, Smiths Detection, Watford, Herts, UK, www.smithsdetection.com). However, there was still the second task to be completed. And molecular fingerprinting methods proved to be a suitable tool to explore microbial communities. Hence, different published protocols were evaluated, modified and combined to develop an appropriate method to study diversity and distribution of *Bacillus* species in soil.

Overall, the following can be concluded from our findings:

- Anthrax was once a common disease in Switzerland and there are a considerable number of potentially contaminated sites in the canton of Zurich. Contamination of these sites could be verified in future studies.
- Terbium fluorescence is an adequate method for the detection of bacterial endospores in environmental samples. Many commercial devices for first responders are based on this method.
- Fourier transform infrared spectroscopy (FTIR) is an adequate method for the detection and identification of specific organisms or biological contamination in general (proteins). A library data base or other reference is required. These are limited and often associated with a specific instrument. Commercial FTIR devices for first responders are available, but for chemicals and biological contamination (presence of protein) only. For future work new libraries could be created and established ones extended. Adaptation of data processing and sample preparation may overcome matrix and mixture effects.
- Culture based methods are time-consuming and limited to culturable species. Application of chromogenic agars is less hazardous than conventional culture media but still needs experience.
- Molecular methods are applicable for specific detection. They are faster than culturing but require more analysis time than physico-chemical methods. Easily operated devices applicable for field use are rarely available. To study microbial communities molecular methods seem to be most adequate. Problems to be solved in future work are matrix effects, DNA extraction from and adequate probes for endospores.

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Appendix

Sampling Site

Geography/Geology: The sampling site is located in the surroundings of Zurich. It is situated at about 620 m.a.s.l., is flat and has slight slope (max. 5%) with exposition north-east. It is situated on a Würm moraine.

Soil: Soil is classified as landfill (Fig. 1). Adjacent soil type is a Cambisol (brown soil). The layer relevant for plant growth is of moderate depth (50 – 100 cm). And the soil is characterized by stagnant moisture.

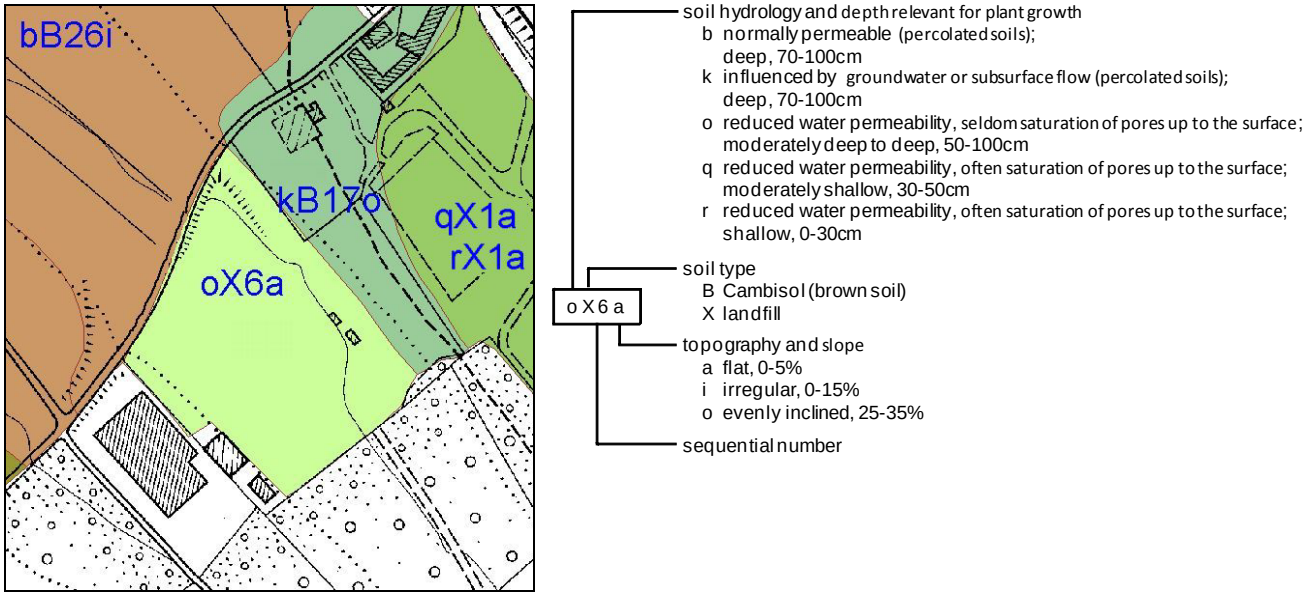


Fig. 1: Soil types at and around the sampling site. (Source: Canton of Zurich unit for soil protection, www.gis.zh.ch,)

Vegetation: Grassland, with a composition typical for Swiss midland. South-west and south-east it is bordered by a forest, at least at the edge mainly coniferous (spruce and fir). North-west there is a graveled dirt road.

Use: The sampling site is a former carcass disposal site, which is nowadays used as riding ring and for show jumping. In direction south-west there is a indoor riding hall.

Sampling: Sampling points are located on a 100 m transect (every 10 m) and on a 15 m line rectangular to it, in parallel to the edge of a forest (every 5 m), forming a T-shaped sampling scheme (Fig. 2). Soil cores with a maximum length of 25 cm were obtained and cut in sections of 5 cm.

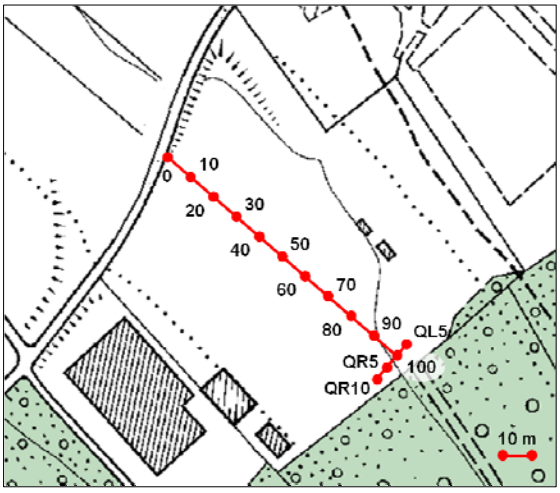
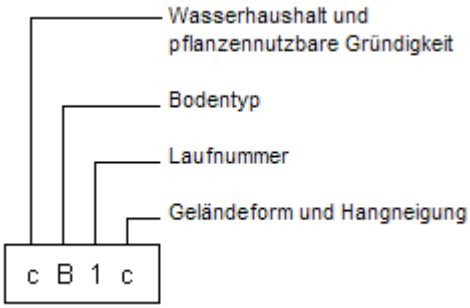


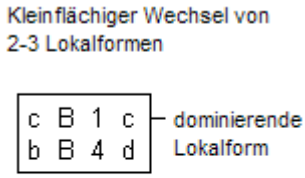
Fig. 2: Sampling site and sampling points. At each point soil cores were taken to a maximal depth of 25 cm. Cores were devided into samples of 5 cm.

Kartencode

Reine Bodeneinheit



Komplex



Wasserhaushalt und pflanzennutzbare Gründigkeit

Wasserhaushaltsklasse (Vernässungsart)	**Pflanzennutzbare Gründigkeit (cm)** sehr tiefgründig	tiefgründig	mässig tiefgründig	ziemlich flachgründig	flachgründig	sehr flachgründig	**Wasserhaushaltsgruppe** (Vernässungsgrad)
	100	70	50	30	10		
senkrecht durchwaschen	a	b	c	d	e		normal durchlässig
	-	f	g	h	i		stauwasserbeeinflusst
	-	k	l	m	n		grund- oder hangwasserbeeinflusst
stauwasser-geprägt	-	o		p		-	selten bis zur Oberfläche porengesättigt
	-	-	-	q	r		häufig bis zur Oberfläche porengesättigt
grund- oder hangwasser-geprägt *	-	s	t	u		-	selten bis zur Oberfläche porengesättigt
	-	-	v	w		-	häufig bis zur Oberfläche porengesättigt
	-	-	-	x	y		meist bis zur Oberfläche porengesättigt
	-	-	-	-	-	z	dauernd bis zur Oberfläche porengesättigt

* blau = mineralische Böden, rosa = organische Böden

Bodentyp

A Aueboden
B Braunerde
E Saure Braunerde
F Fluvisol
G Fahlgley
I Pseudogley
K Kalkbraunerde
M Moor
N Halbmoor
O Regosol
R Rendzina
T Parabraunerde
V Braunerde-Gley
W Buntgley
X Auffüllung
Y Braunerde-Pseudogley

Geländeform und Hangneigung (%)

a	eben	0 - 5
b	eben	5 - 10
c	konvex	- 10
d	konkav	- 10
e	ungleichmässig	0 - 10
f	gleichmässig geneigt	10 - 15
g	konvex	- 15
h	konkav	- 15
i	ungleichmässig	0 - 15
j	gleichmässig geneigt	15 - 20
k	gleichmässig geneigt	20 - 25
l	konvex	- 25
m	konkav	- 25
n	ungleichmässig	0 - 25
o	gleichmässig geneigt	25 - 35
p	konvex	- 35
q	konkav	- 35
r	ungleichmässig	0 - 35
s	gleichmässig geneigt	35 - 50
t	konvex	- 50
u	konkav	- 50
v	ungleichmässig	0 - 50
w	gleichmässig geneigt	50 - 75
x	ungleichmässig	0 - 75
y	gleichmässig geneigt	> 75
z	ungleichmässig	0 - > 75

http://www.oie.int/wahis/public.php?page=disease

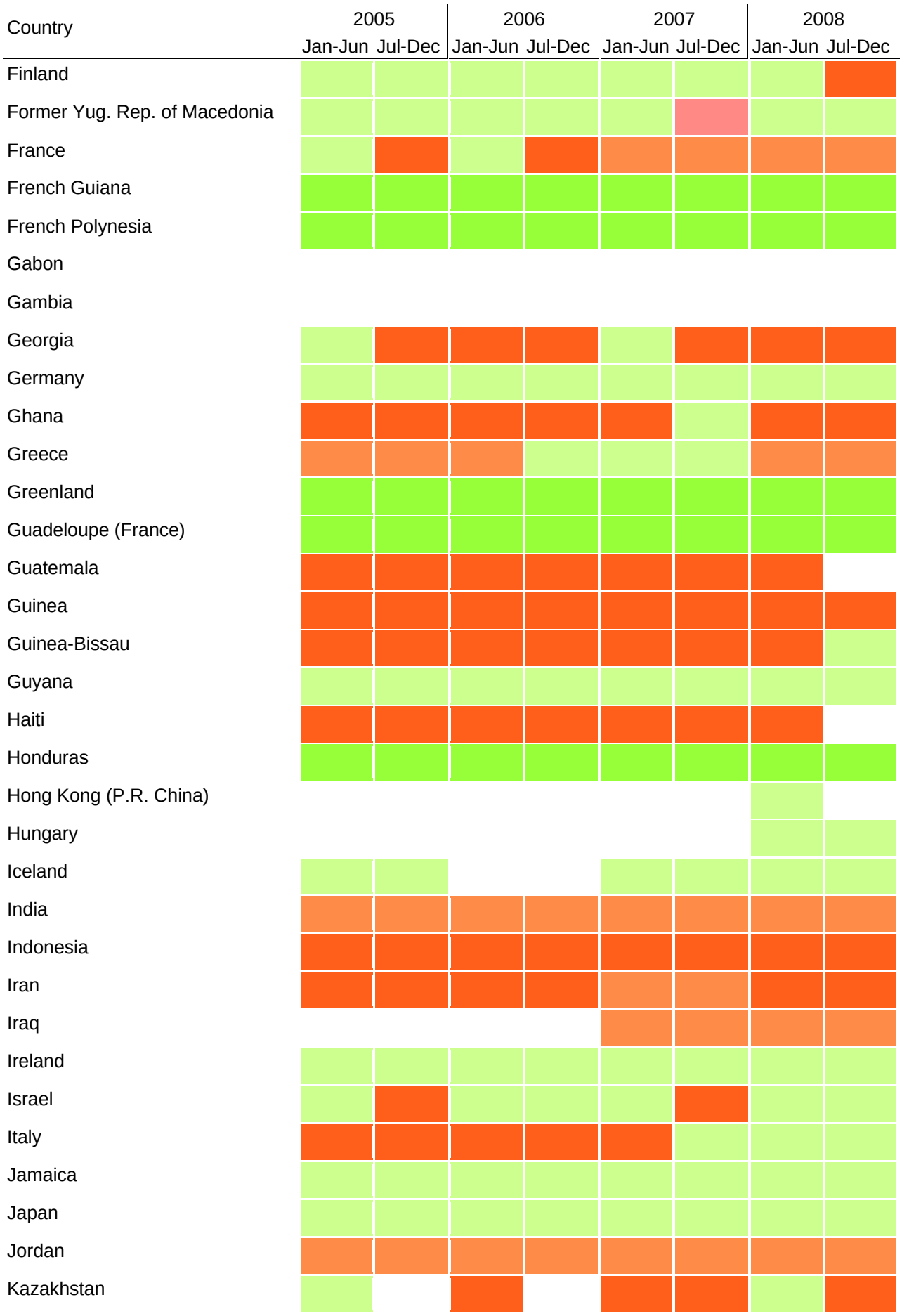
Disease timelines: Anthrax

Key to colours

There is no information available on this disease
Never reported
Disease not reported during this report period
Disease suspected but not confirmed
Confirmed infection but no clinical disease
Confirmed clinical infection
Confirmed infection but limited to certain zones

Country	2005		2006		2007		2008	
	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec
Brazil								
Brunei Darussalam								
Bulgaria								
Burkina Faso								
Burundi								
Cambodia								
Cameroon								
Canada								
Cayman Islands								
Central African Republic								
Chad								
Chile								
China (People's Rep. of)								
Chinese Taipei								
Colombia								
Congo (Dem. Rep. of the)								
Congo (Rep. of the)								
Costa Rica								
Cote D'Ivoire								
Croatia								
Cuba								
Cyprus								
Czech Republic								
Denmark								
Djibouti								
Dominican Republic								
Ecuador								
Egypt								
El Salvador								
Eritrea								
Estonia								
Ethiopia								
Fiji								

Anthrax in the world



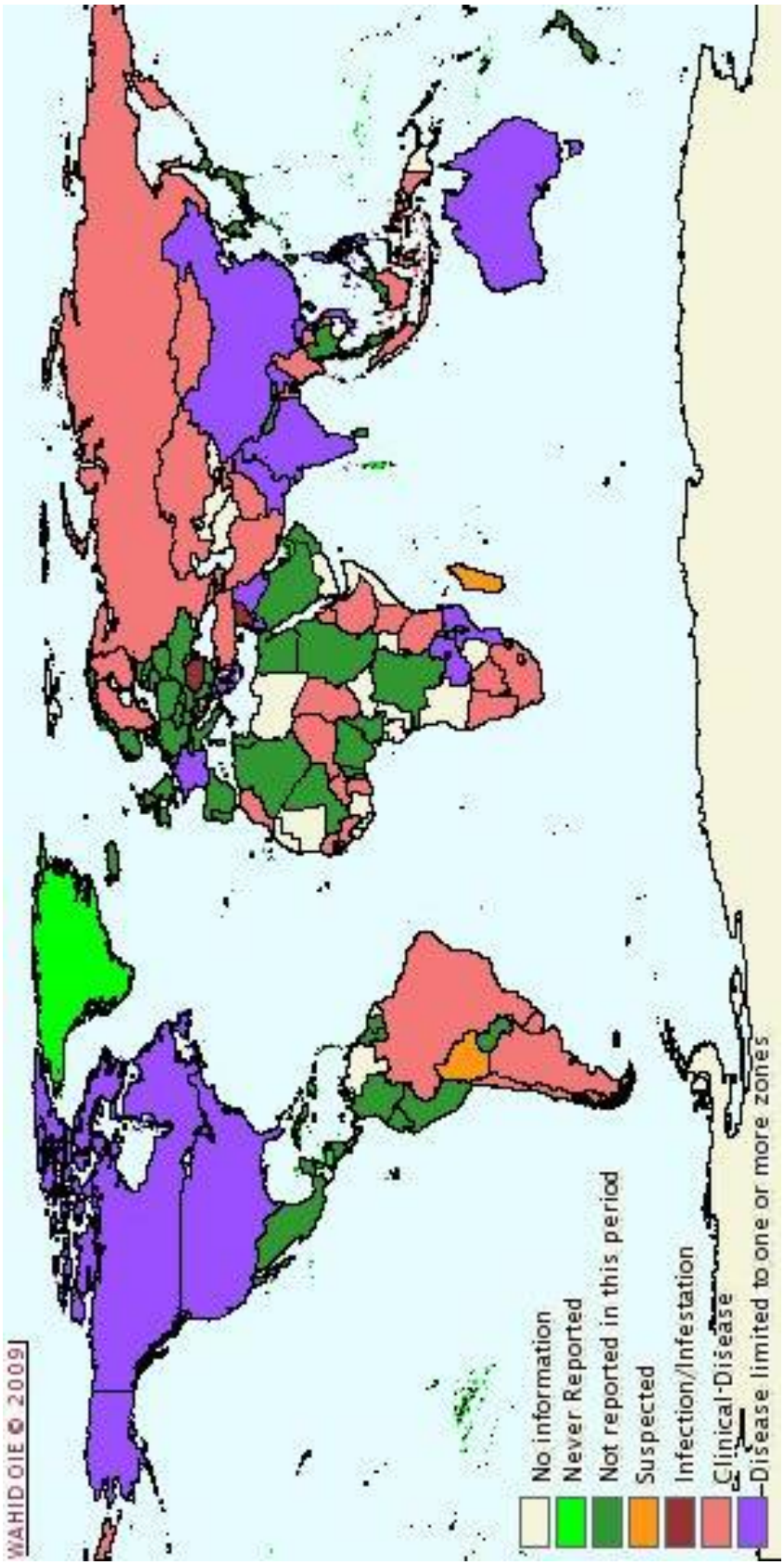

Country
2005
2006
2007
2008
Jan-Jun
Jul-Dec
Jan-Jun
Jul-Dec
Jan-Jun
Jul-Dec
Jan-Jun
Jul-Dec
Kenya
Korea (Dem. People's Rep.)
Korea (Rep. of)
Kuwait
Kyrgyzstan
Laos
Latvia
Lebanon
Lesotho
Libya
Liechtenstein
Lithuania
Luxembourg
Madagascar
Malawi
Malaysia
Maldives
Mali
Malta
Martinique (France)
Mauritania
Mauritius
Mexico
Micronesia (Federated States)
Moldavia
Mongolia
Montenegro
Morocco
Mozambique
Myanmar
Namibia
Nepal
Netherlands

Anthrax in the world

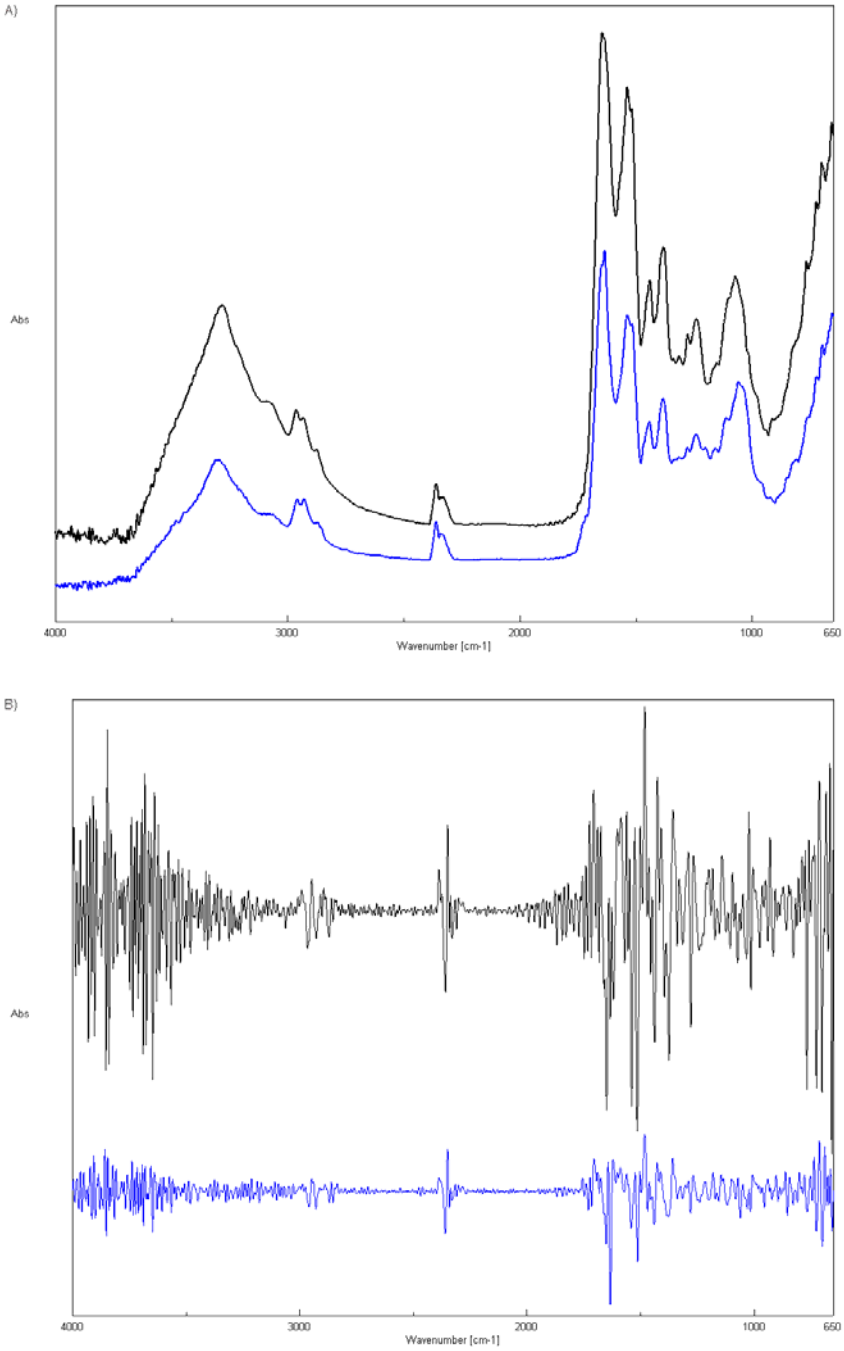

Country
2005
Jan-Jun
Jul-Dec
2006
Jan-Jun
Jul-Dec
2007
Jan-Jun
Jul-Dec
2008
Jan-Jun
Jul-Dec
Sudan
Suriname
Swaziland
Sweden
Switzerland
Syria
Tajikistan
Tanzania
Thailand
Togo
Trinidad and Tobago
Tunisia
Turkey
Uganda
Ukraine
United Arab Emirates
United Kingdom
United States of America
Uruguay
Uzbekistan
Vanuatu
Venezuela
Vietnam
Wallis and Futuna Islands
Yemen
Zambia
Zimbabwe

http://www.oie.int/wahis/public.php?page=disease

Disease distribution map: Anthrax (July – December 2008)

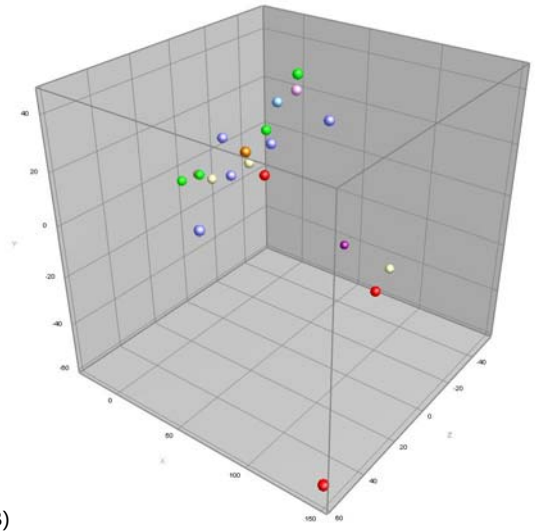


Additional Tables & Figures

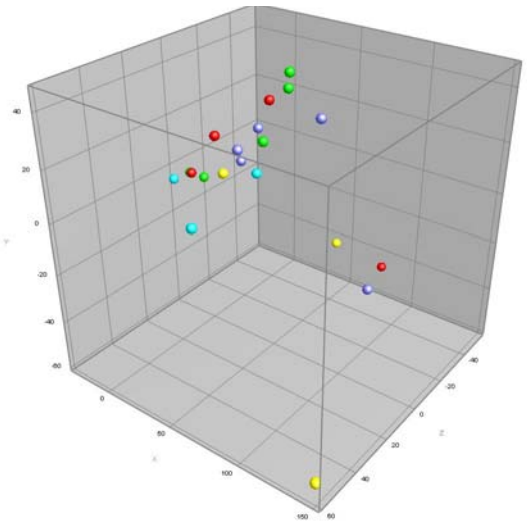


Typical FTIR absorption spectra of *B. atrophaeus* (black line) and *B. subtilis* (blue line).
A) original spectra, B) second derivative.

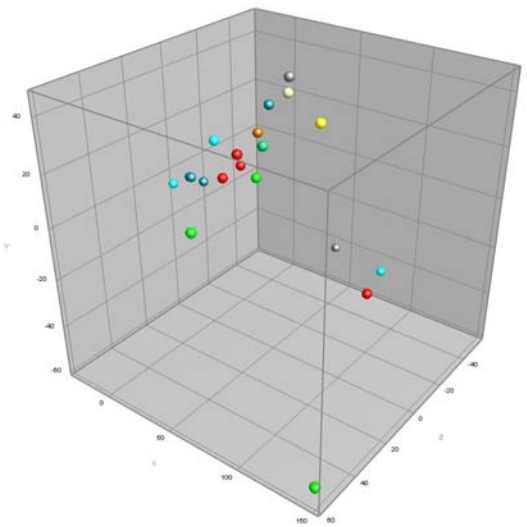
A)



B)

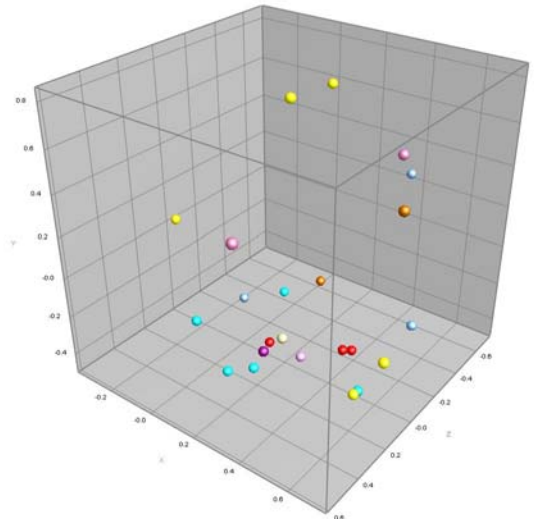


C)

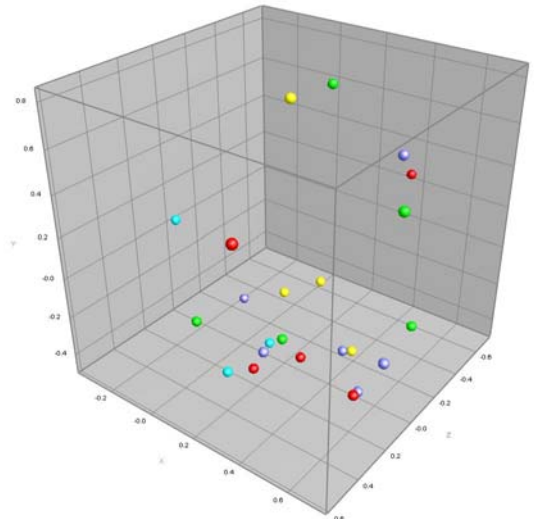


Principal Components Analysis (PCA) on the band matching table. First three components are shown, colours are groups.
A) group = sampling position, B) group = sampling depth, C) group = content of organic carbon

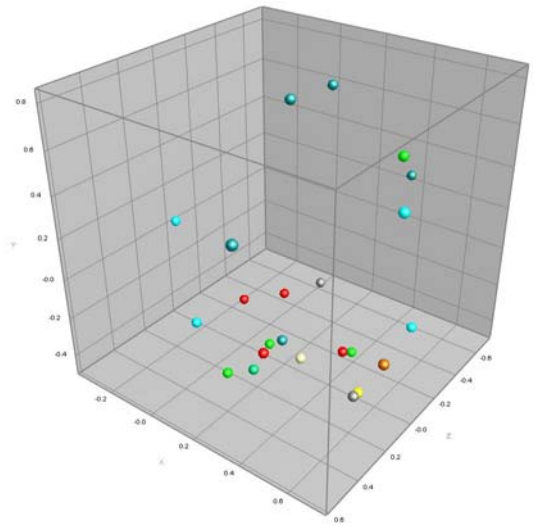
A)



B)



C)



Multi-Dimensional Scaling (MDS) on the band matching table. First three components are shown, colours are groups.
A) group = sampling position, B) group = sampling depth, C) group = content of organic carbon

TTGE band matching table

sample	total bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0_0	5				■	■		■	■			■							
0_5	0																		
0_10	2				■	■													
0_15	0																		
0_20	1		■																
10_0	1		■																
10_5	5		■		■	■		■			■								
10_10	1		■																
20_0	7		■		■	■		■	■		■	■							
20_5	1		■																
30_0	1		■																
30_5	7		■		■	■		■	■		■	■							
30_10	1		■																
30_15	1		■																
30_20	1		■																
40_0	1		■																
40_5	1		■																
40_10	6		■		■	■	■	■		■									
40_15	1		■																
50_0	1		■																
50_5	1		■																
50_10	1		■																
50_15	1		■																
60_0	1		■																
60_5	1		■																
60_10	1		■																
60_15	1		■																
70_0	1			■															
70_5	1		■																
70_10	1		■																
70_15	1		■																
70_20	1		■																
80_0	1		■																
80_5	1		■																
80_10	1	■																	
80_15	1	■																	
90_0	1	■																	
90_5	8	■			■	■					■	■				■	■	■	
90_10	3		■								■	■							
100_0	1			■															
100_5	1		■																
100_10	0																		
QL5_0	1		■																
QL5_5	0																		
QL5_10	1			■															
QL5_15	5		■			■					■					■		■	
QR5_0	6		■		■			■	■		■				■				
QR5_5	3		■		■				■										
QR5_10	7				■	■		■	■		■	■	■						
QR5_15	4		■			■				■		■							
QR5_20	6		■		■				■	■	■			■					
QR10_0	1			■															
QR10_5	1		■																
QR10_10	11		■		■	■	■		■		■	■	■		■	■	■		
QR10_15	14		■		■	■	■		■	■	■	■	■	■	■		■	■	■
QR10_20	5		■		■		■				■	■							

Posters

Occurrence and frequency of bacterial spores in soils determined by a fluorescence method

Andrea Brandes[a], Linda Kölle[b] & Helmut Brandl[a]

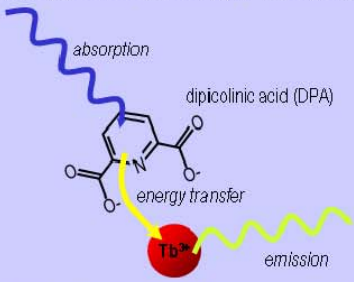
[a]University of Zurich, Institute of Environmental Sciences, [b]University of Applied Sciences, Wädenswil

Intro

The goal of the project was to determine the occurrence of bacterial spores in soil. As unique chemical features, bacterial spores contain high amounts of dipicolinic acid (DPA) which facilitates their identification in natural environments.

Methods

In the presence of terbium, DPA forms a complex (Tb-DPA-chelate) showing a distinct fluorescence spectrum as (**Fig 1**).



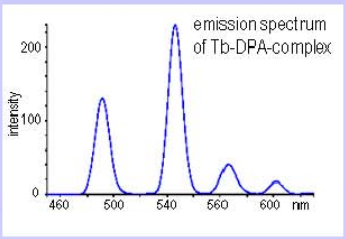


Fig. 1. Fluorescence of Tb-DPA-complex

Samples were collected from grassland, former tannery locations, forest soil, and fluvial sediment. After lyophilization, standard addition with *Bacillus subtilis* spores was performed to determine the overall spore number (assessed as *B. subtilis* equivalents) after autoclaving to release DPA. Terbium chloride was added after filtration and its fluorescence determined.

Results

The detection limit of spores in aqueous solutions was 1.7×10^4 *B. subtilis* spores per mL. Grassland soil contained higher spore numbers (1 to 5×10^8/g soil) than forest soil (approx. 10^7/g soil) (**Fig. 2**).

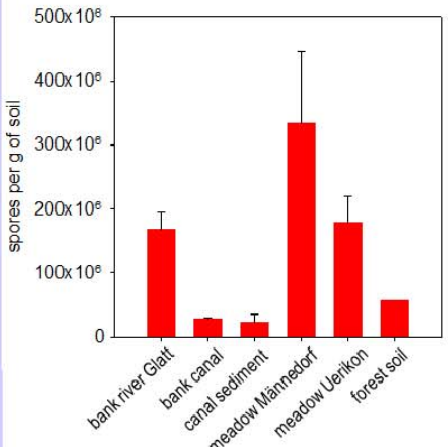


Fig. 2. Spore content of different soils (expressed as equivalents of *B. subtilis*); n=3

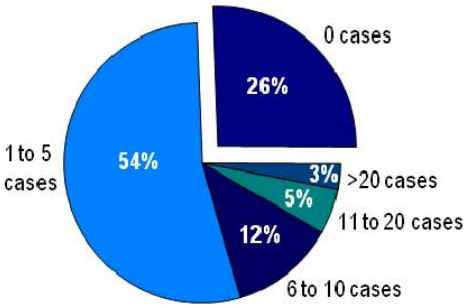


Fig. 3. Historical cases of anthrax in the Canton of Zürich between 1878 and 1919 (in % of total 171 municipalities).

Perspectives & Application

The method will be used as fast screening to investigate location with potentially high bacterial soil-borne spore concentrations such slaughter houses, tanneries, weaving mills, spinning mills, or carcass disposal sites which will be mapped in a land register ("Kataster"). From a historical point of view, there have been 667 cases of anthrax in the Canton of Zürich between 1878 and 1919 (**Fig. 3**). At several sites, carcasses have been buried in soil. These sites are of potential interest for further investigation.

Conclusions

Standard solutions of *B. subtilis* spores suspended in water were easily determined. Compared to aqueous samples, the investigation of soil showed certain drawbacks (chemical interferences). A combination of luminescence measurements with a chromatographic separation (solid phase extraction) of DPA is suggested. To determine soil-borne spore distribution in full, small-scale soil sampling (including depth profiles) is needed.

Poster Swiss Microbial Ecology Meeting 2004, Neuchâtel

Historical cases of animal anthrax in Switzerland

Incidents in the canton of Zurich between 1878 and 1919

Andrea Brandes & Helmut Brandl

University of Zurich, Institute of Environmental Sciences

Intro & Methods

- Animal anthrax occurred frequently 100 years ago, whereas toady it seems rather "exotic"
- Historical records from the cantonal veterinary office (registers of epizootics between 1878 and 1919) were analyzed and evaluated on the level of political communities.
- Relevant ancient industrial activities (slaughterhouses, tanneries, fur-manufacturing industries, weaving mills, and spinneries) in all 171 communities of the canton of Zurich were investigated (cantonal registry of commercial enterprises from 1842 to 1870)
- Meteorological data (monthly mean temperature and wet precipitation) were obtained from MeteoSchweiz

Results

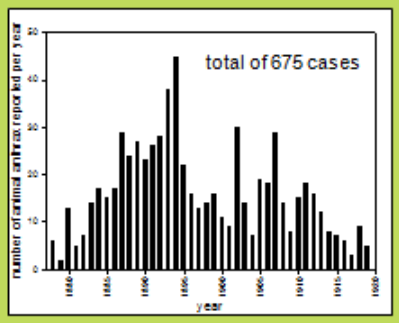


Fig. 1: Animal anthrax in the canton of Zurich involving 642 cattle (93.7%), 21 horses (3.1%), 16 pigs (2.3%) and 6 goats (0.9%)

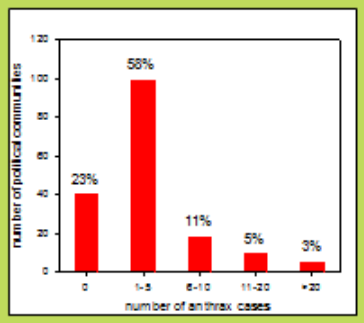


Fig. 2: Cases of animal anthrax in the 171 communities of the canton of Zurich between 1878 and 1919

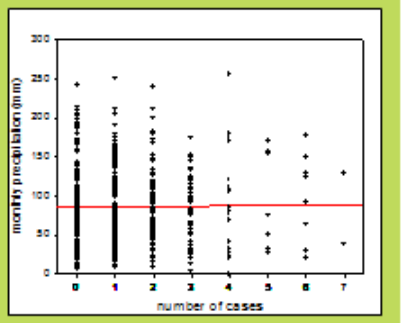


Fig. 3: Monthly number of anthrax cases in relation to mean monthly precipitation between 1878 and 1919

Correlation between occurrence of cases in a community and local industries

(ANOVA with χ^2 test to test significance)

- wool processing companies → significant (P=0.004)
- tanneries → significant (P=0.032)
- horse hair mills → not significant (P=0.914)

In the communities reporting the highest numbers of cases, tanneries, wool processing industries and/or horse hair mills were situated.

Discussion & Infos

- industrialization and trade favored the spread of anthrax.
- vaccination of animals and disinfection of possibly contaminated raw material before import led to a decrease of cases.
- prohibition to bury dead animals represents a major contribution to the reduction of animal anthrax cases in industrialized countries.
- in contrast to other reports, no statistical relationship between the number of cases and meteorological data (wet precipitation, temperature) was found.

Some background info about anthrax

- well-known infectious disease occurring in wild animals and livestock
- spore-forming, Gram-positive bacterium *Bacillus anthracis*, extremely resistant
- widespread soil bacterium, occurs worldwide, in some parts endemic (Asia, central Africa, southern and South America)

Poster Schweizerische Gesellschaft für Mikrobiologie, Swiss Society for Microbiology, annual congress 2006, Lausanne

Detection and identification of bacterial spores in environmental samples with Fourier transform infrared (FT-IR) spectroscopy

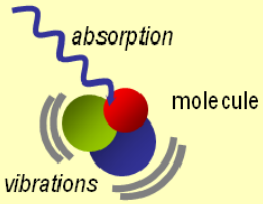
Andrea Brandes & Helmut Brandl
University of Zurich, Institute of Environmental Sciences

Intro & Methods

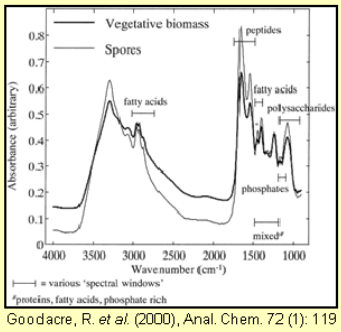
- **Fourier transform infrared (FT-IR) spectroscopy:** rapid and non-invasive method to detect and identify microorganisms, at present possible for pure cultures only
- **Aim of this study:** develop a fast and reproducible non-molecular method to identify bacteria in environmental samples
- **Sporeproduction** of 5 *Bacillus* species (*B. atrophaeus, B. brevis, B. circulans, B. lentus, B. thuringiensis*): cultivation in identical growth medium, same conditions, transfer to sporulation medium, harvest by centrifugation, washing and subsequent freeze drying

Principle of FT-IR spectroscopy

- Absorption of light (IR) leads to vibrations in molecules
- Through a mathematical transformation the sum of all vibrations can be made visible as a spectrum
- Each molecule/substance/organism shows a specific FTIR spectrum



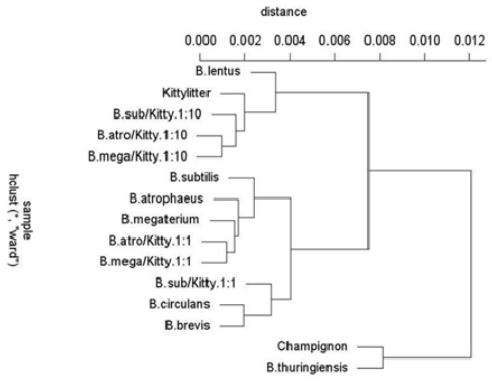
Spectrum of a *Bacillus subtilis*

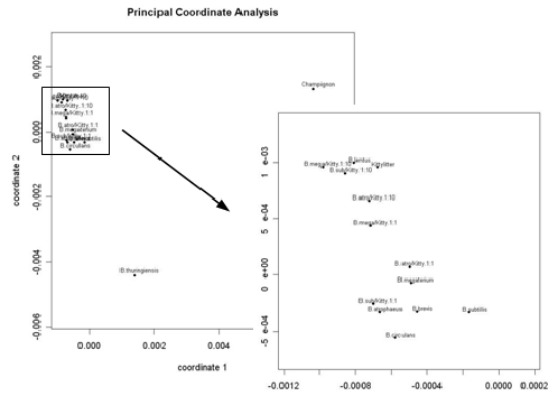


Goodacre, R. *et al.* (2000), Anal. Chem. 72 (1): 119

Results

- Clusteranalysis and multidimensional scaling (principal coordinates analysis) of the spectra lets discriminate different species and spores-matrix-mixtures





Discussion

- Preliminary evaluation of spectral data showed distinct differences between the different Bacillus species.
- Differences might serve as a basis for species differentiation and detection in environmental samples
- A standard library of spectra was created. After further extention it is intended to be used to identify mixtures of matrix compounds and bacterial species
- First experiments with model matrices (e.g. kitty litter) have been started and will be continued with soil

Diversity of *Bacillus* species in soil

Andrea Brandes & Helmut Brandl

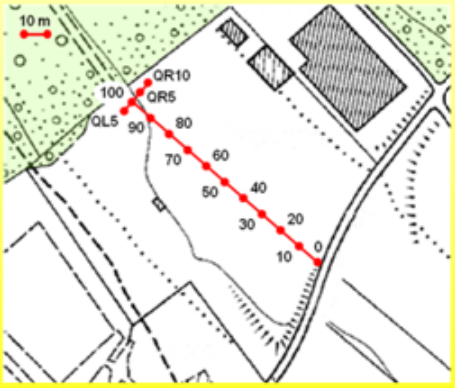
University of Zurich, Institute of Environmental Sciences

Intro & Methods

Bacillus sp. are common soil bacteria which are able to form endospores under unfavorable environmental conditions. These spores are extremely resistant to environmental stress and found to survive in soil for many decades. Most *Bacillus* sp. are harmless to humans and animals, but some of them (e.g. *Bacillus anthracis*) can cause severe diseases like anthrax.

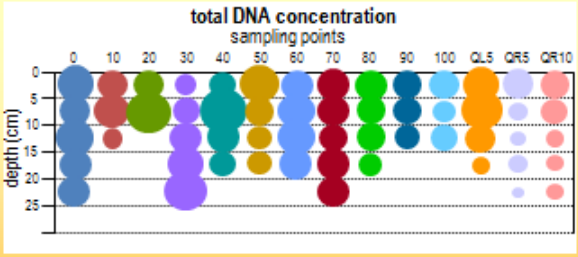
The aim of this study was the detection of differences in the *Bacillus* diversity along a soil transect, at different depth and at different sampling sites (e.g. "native" soil vs. soil possibly contaminated with anthrax). **Does the presence of a specific species influence the occurrence of other, common *Bacillus* species?**

Samples were collected from a meadow adjacent to a forest, formerly used as carcass burial site. Whole DNA was extracted directly from the soil samples.



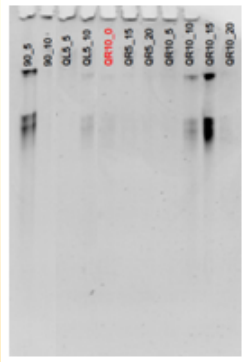
After PCR with primers for a *Bacillus* specific 16S rDNA sequence (hypervariant region, HV; highly specific for strains), samples were analyzed with temperature gradient gel electrophoresis (TTGE).

Preliminary Results



- Total amount of extracted soil-DNA differs at different sampling points: Near the forest it seems to be lower.
- The concentration of whole DNA also tend to decrease with sampling depth.

- TTGE shows the presence of *Bacillus* species in the soil investigated. However, on the first sight there are hardly differences between samples.
- The first band probably represents singe stranded DNA.



Discussion

- Preliminary results demonstrate, that methods and evaluated parameters (primers, PCR-protocol, TGGE-protocol) are applicable.
- More diversity between samples was expected. Further experiments will prove if the occurrence of *Bacillus* species in soil is in fact that homogenous.
- We already showed with a terbium fluorescence method (data not shown), that forest soil contains less bacterial spores than soil from beneath a meadow. This supports the result of less DNA in samples from the edge of the forest.
- The decrease of DNA with depth could be interpreted as follows: At the surface are more soil influencing factors (temperature, humidity, nutrient input etc.), that means more potential niches. Another interpretation could be the soil compression which increases with depth and allows less (micro) cavities.

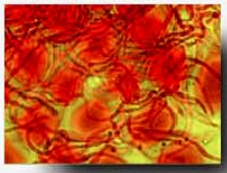
Infrared spectroscopy as a tool for the rapid detection of fungal endophytes in infected grasses

Helmut Brandl, Andrea Brandes Ammann, Johanna Reiner
University of Zurich, Institute of Environmental Sciences

infected leaves (*Neotyphodium*)

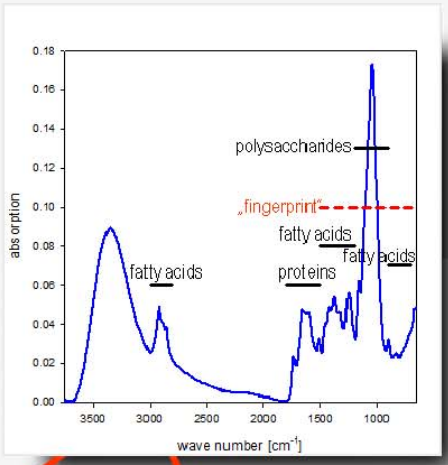
non-infected seeds

infected seeds



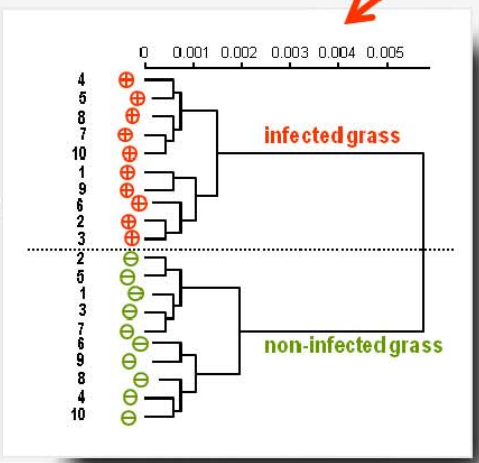
1) harvesting
2) drying (12 h @ 65°C)
3) grinding to powder
4) collection of IR spectra (ATR, ZnSe)
5) chemometrical data analysis

An infrared spectrum is a "fingerprint" of a sample indicating vibrations of chemical bonds. Because each material is a unique combination of atoms, infrared spectroscopy (FTIR) can result in a positive identification of every different kind of material!

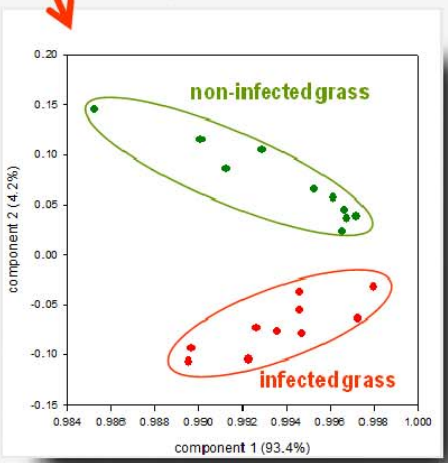


Spectra were analyzed chemometrically resulting in a rapid detection of fungal infections in plants.

hierarchical cluster analysis



factor analysis



In summary, FTIR proved to be a powerful tool for the identification and differentiation of microorganisms !!!

Acknowledgements

Dank

Dank

Als erstes möchte ich mich bei *Helmi Brandl* bedanken, der mir als Betreuer immer mit Rat und Tat zur Seite stand. Deine Ideen und Inputs waren immer sehr willkommen und hilfreich während meiner Arbeit, genau so wie die praktische Unterstützung.

Dann gilt mein Dank auch *Daniel Fischer*, der mir diese Dissertation neben der Arbeit bei der Fachstelle Biosicherheit überhaupt erst ermöglichte.

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Gedankt sei auch allen anderen, die mich in irgend einer Weise während meiner Doktorarbeit unterstützten.

Vielen, ♡-lichen Dank!

Andrea

Curriculum Vitae

Curriculum Vitae

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Ausbildung

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DIPLOMARBEIT 1996	*„Der Einfluss von Landschaftsstrukturen auf die Nutzung von Kulturflächen durch Wildtiere - ein Vergleich zweier Gebiete im Kanton Thurgau“* ETH Zürich, Eidg. Forschungsanstalt für Wald, Schnee und Landschaft WSL
DISSERTATION 2003-2009	*"Diversity of the genus Bacillus in soil and the occurrence of bacterial endospores in soil, with special reference to Bacillus anthracis"* Universität Zürich, Institut für Umweltwissenschaften

Berufliche Tätigkeiten während des Promotionsstudiums

Seit 1999	Baudirektion Kanton Zürich, AWEL Amt für Abfall, Wasser, Energie und Luft, Abteilung Abfallwirtschaft und Betriebe, Sektion Biosicherheit Wissenschaftliche Mitarbeiterin (60 – 70%)